

Docket No.: G0744.70042US07  
(PATENT)

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

Applicant: Katherine Gordon et al.  
Serial No.: 07/839,194  
Confirmation No.: 6108  
Filed: February 20, 1992  
For: TRANSGENIC ANIMALS SECRETING DESIRED PROTEINS INTO MILK  
Examiner: D. A. Montanari  
Art Unit: 1632

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I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office electronic filing system in accordance with § 1.6(a)(4).

Dated: June , 2008

Signature: /Nicole Millette Hawes/

**DECLARATION OF HARRY M. MEADE**

Mail Stop Amendment  
Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

Dear Sir:

I, Harry M. Meade, declare that:

1. I am the Senior Vice President of Research and Development with GTC Biotherapeutics, Inc., the exclusive licensee of the present application, which is a company I joined in ~~1994~~ and of which I am a founding member. I direct all transgenic molecular biology research and development efforts conducted within GTC Biotherapeutics, Inc., including the evaluation of technologies having the potential to increase the efficiencies of transgenic protein expression. 1993 HM

2. I have over 25 years of research experience, the last at least fifteen years pertaining to transgenic research and the development of protein expression systems. I have been recognized as a pioneer in the field.

3. I have also held scientific positions with Genzyme Corporation, Biogen and Merck. I received my Ph.D. in Biology from the Massachusetts Institute of Technology and completed post-doctoral studies at Harvard University. Further details of my education and research experience, including lists of my publications, patents and lectureships, are found in my *curriculum vitae* (Exhibit A).

4. I make this declaration in support of the above-referenced patent application and in response to the Final Office Action of May 3, 2007. I understand that this application was filed on February 20, 1992 and claims priority to an application filed on April 9, 1986.

5. As of April 9, 1986, the use of proteins, such as human proteins, for medical purposes had been common for several years. Up until the advances afforded with recombinant DNA technology, the supply of human proteins, which were generally derived from human plasma and tissue, had been extremely limited. With recombinant DNA technology, other methods of producing proteins, such as in cell culture systems, became available. However, expression of proteins in cell culture proved to have drawbacks. Microorganisms, such as yeast and bacteria, generally were not suitable for mammalian protein production due to their inability to provide the same posttranslational modifications as in mammalian cells, and commercial-scale protein production in cell cultures, such as mammalian cell cultures, was very expensive.

6. As of April 9, 1986, several investigators had attempted to overcome those problems by creating transgenic animals containing a "foreign gene" encoding the desired protein. The present application discusses some of those attempts. Some scientists had made transgenic animals where "the foreign genes are expressed in specific tissues" by using a tissue-specific promoter (page 1). Some had made transgenic animals where "the foreign gene is transcribed only during a certain time period" (page 1). These early transgenic animals, however, did not avoid all of the problems with existing mammalian protein production technology. For example, the proteins encoded by the foreign genes were not expressed in a medium from which they could readily be obtained and purified.

7. On page 2 of the present application, it is described that:

In general, the invention features a DNA sequence containing a gene encoding a protein, the gene being under the transcriptional control of a mammalian milk protein promoter which does not naturally control the transcription of the gene... The promoter can be that of a milk serum protein or a casein protein, although milk serum proteins are preferred, as will be discussed in more detail below.

Further, on pages 2 and 3 of the present application, it is described that:

Secretion of the protein into the host mammal's milk facilitates purification and obviates removal of blood products and culture media additives, some of which can be toxic or carcinogenic. More importantly, protein yields will be high and production will be more cost effective and efficient.

8. The present application also provides an example in which the mouse WAP promoter controls expression of human uterine t-PA (pages 9-11) and an example in which the mouse WAP promoter controls the expression of hepatitis B surface antigen (pages 11-13).

9. As a result of the invention of the above-referenced patent application, a powerful alternative method for protein production was provided, the importance and value of which was recognized by those of ordinary skill in the art as well as those in the pharmaceutical industry. The inventors published a paper containing the above-mentioned examples in 1987. See Gordon et al. (1987), *Biotechnology* 5: 1183-1187 (Exhibit B). This was the first paper to describe the production of a recombinant human protein in milk. Castro et al. (1999), *Genetic Analysis: Biomolecular Engineering* 15: 179-187 (Exhibit C) and Clark et al. (1998), *Journal of Mammary Gland Biology and Neoplasia* 3: 337-350 (Exhibit D). It has been frequently cited by subsequent papers and has been characterized as having "started a mini-revolution" and having founded an entire industry. See, e.g., Wall (1999), *Livestock Production Science* 59: 243-255 (Exhibit E) and Wall et al. (1997), *Journal of Dairy Science* 80: 2213-2224 (Exhibit F). A new industry was rapidly formed subsequent to the aforementioned 1987 publication. See, e.g., Wall (1999), *Livestock Production Science* 59: 243-255 (Exhibit E) and Wall et al. (1997), *Journal of Dairy Science* 80: 2213-2224 (Exhibit F). Additionally, as a result of the invention of the above-referenced patent application, hundreds of lines of transgenic mice, sheep, goats, pigs and bovine that express recombinant proteins in their milk have been produced (See, e.g., Castro et al. (1999), *Genetic Analysis: Biomolecular Engineering* 15: 179-187 (Exhibit C) and Wall (1999), *Livestock Production Science* 59: 243-255 (Exhibit E)) and several

biotechnology companies developed (See, e.g., Lubon (1996), *Transfusion Medicine Reviews* X(2): 131-143 (Exhibit G)), an example of which is GTC Biotherapeutics, Inc.

10. I have read the above-referenced patent application as well as the Final Office Action of May 3, 2007 and disagree with the Examiner's characterization of the term "naturally" as recited in the claims. As pointed out in the "Background of the Invention" section of the above-referenced patent application, in a number of instances, it has been possible to utilize promoters and associated regulatory segments from one gene to control transcription of the coding sequence from another and obtain specific expression of the protein in tissues appropriate to the regulatory sequences. In fact, there existed, at the time of the filing of the above-referenced patent application, experimental data indicating that certain regulatory sequences which control expression of a particular protein could also be used to control expression of completely unrelated proteins (i.e., proteins not associated with the promoter as found in nature) through the construction of hybrid genes.

11. The above-referenced patent application importantly provides that milk protein promoters can also be used in the expression of unrelated proteins. This is exemplified in the above-referenced patent application with the expression in mouse mammary gland of two proteins, hepatitis B surface antigen and tissue plasminogen activator, both of which are unrelated to the milk protein promoter used, the whey acid promoter. In light of the teachings of the above-referenced patent application and the basic level of understanding and knowledge in the art, it would be understood that a promoter which "does not naturally control transcription of a gene" is one that is unrelated to (i.e., not in nature associated with) the gene. In other words, it would be understood that such milk protein promoter controls transcription of a heterologous gene. It would have been clear to one of ordinary skill in the art, if they had read the present application on April 9, 1986 that a "milk protein promoter sequence which does not naturally control transcription" of a gene, meant a milk protein promoter sequence that in nature (i.e., absent genetic engineering) did not control transcription of the gene.

12. This is further illustrated by comparing the application's discussion of promoters and signal sequences. The application encompasses any promoters "naturally associated with any protein which is normally secreted into mammalian milk" (pages 3-4). Similarly, the application encompasses any signal sequences "naturally associated with the desired protein, if the protein is

normally secreted (e.g., t-PA).” (page 6). One of ordinary skill in the art would clearly have understood this to mean that the t-PA signal sequence could be used to secrete t-PA. For the same reason, one of ordinary skill in the art would have understood the parallel promoter language to mean that the inserted gene’s promoter would not be used. For example, the albumin promoter would not be used if albumin were the inserted gene because the albumin promoter naturally controls the transcription of the albumin gene.

13. One of ordinary skill in the art would not reasonably interpret the milk protein promoter of the claims to be one that does not by nature want to control transcription as the Examiner has suggested. The Examiner states that a promoter by its very nature controls transcription, so a promoter always naturally controls transcription of the gene it is promoting (with or without genetic engineering). This interpretation, besides being contrary to what one of ordinary skill in the art on April 9, 1986 would have understood, as I discussed above, would have been nonsensical to one of ordinary skill in the art. It would also render “naturally” in the “naturally controls the transcription” wholly redundant. Such an interpretation is not reasonable and, as the Examiner has himself pointed out, counterintuitive.

14. As discussed above, the application discusses attempts to produce medically desired proteins on a large scale. The milk system, including milk protein promoters, allowed this, and was simply a tool for doing so. The application makes clear that the invention was intended to produce large quantities of medically important proteins, such as “proteins useful in the treatment, prevention, and/or diagnosis of human disease” (page 6).

15. Because of the seminal work described in the above-reference patent application, one of ordinary skill in the art was then able to use milk protein promoters to express proteins they did not naturally control in the milk of transgenic animals, which, as mentioned above, occurred rapidly after the Gordon et al. 1987 publication.

I, the undersigned, declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under §1001 of title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this document and any patent which may issue from the above-identified patent application.

June 3, 2008  
Date

Harry M. Meade  
Harry M. Meade

**BIOGRAPHICAL SKETCH**

|   |  |  |         |                                    |
|---|--|--|---------|------------------------------------|
| NAME<br><b>Harry Meade, Ph.D.</b>   |  | POSITION TITLE<br><b>Senior Vice President of R&amp;D, GTC Biotherapeutics</b> |         |                                    |
| EDUCATION/TRAINING <i>(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)</i> |  |  |         |                                    |
| INSTITUTION AND LOCATION  |  | DEGREE   | YEAR(s) | FIELD OF STUDY                     |
| Union College   |  | B.S.   | 1969    | Chemistry & Electrical Engineering |
| Massachusetts Institute of Technology, Cambridge, MA  |  | Ph.D.  | 1977    | Biology                            |

**A. Positions and Honors****Positions and employment**

- 1977 – 1979 Post doctoral studies in the Cellular and Development Biology Department at Harvard University, Cambridge, MA
- 1979 - 1981 Sr. Scientist in Development Department, Merck & Co, Rahway, NJ
- 1981 – 1990 Scientist and later senior scientist at Biogen, Inc., with a focus on expression systems, first in *Streptomyces* and then in the mammary gland.
- 1990 – present Various positions at Genzyme, starting as a molecular biologist in the transgenics group. Appointed research director in 1993 when GTC became an independent company, Vice President of Transgenic Research in 1994 and Senior VP in 2002.

**B. Publications (out of 41 peer-reviewed papers)**

1. Miroff G, Meade HM, Winetroun M, and Lamberson HV. Unique glycoprotein from mouse milk containing the mammary tumour agent. *Nature*, 227: 1243-4, 1970.
2. Baglioni C, Jacobs-Lorena M and Meade H. The site of action of inhibitors of initiation of protein synthesis in reticulocytes. *Biochim Biophys Acta*, 277: 188-97, 1972.
3. Meade HM and Signer ER. Genetic mapping of *Rhizobium meliloti*. *Proc Natl Acad Sci U S A*, 74: 2076-8, 1977.
4. Meade HM, Long SR, Ruvkun GB, Brown SE, and Ausubel FM. Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon Tn5 mutagenesis. *J Bacteriol*, 149: 114-22, 1982.
5. Meade H, Gates L, Lacy E, and Lonberg N. Bovine alpha S1-casein gene sequences direct high level expression of active human urokinase in mouse milk. *Biotechnology (N Y)*, 8: 443-6, 1990.
6. DiTullio P, Cheng SH, Marshall J, Gregory RJ, Ebert KM, Meade HM, and Smith AE. Production of cystic fibrosis transmembrane conductance regulator in the milk of transgenic mice. *Biotechnology (N Y)*, 10: 74-7, 1992.
7. Rybak SM, Hoogenboom HR, Meade HM, Raus JC, Schwartz D, and Youle RJ. Humanization of immunotoxins. *Proc Natl Acad Sci U S A*, 89: 3165-9, 1992.
8. Ebert KM, DiTullio P, Barry CA, Schindler JE, Ayres SL, Smith TE, Pellerin LJ, Meade HM, Denman J, and Roberts B. Induction of human tissue plasminogen activator in the mammary gland of transgenic goats. *Biotechnology (N Y)*, 12: 699-702, 1994.
9. Gutierrez A, Meade HM, DiTullio P, Pollock D, Harvey M, Jimenez-Flores R, Anderson GB, Murray JD, and Medrano JF. Expression of a bovine kappa-CN cDNA in the mammary gland of transgenic mice utilizing a genomic milk protein gene as an expression cassette. *Transgenic Res*, 5: 271-9, 1996.

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  11. Meade HM. Dairy gene. *Sciences (New York)*, 37: 20-5, 1997.
  12. Gavin WG, Pollock D, Fell P, Yelton D, Cammuso C, Harrington M, Lewis-Williams J, Midura P, Oliver A, Smith TE, Wilburn B, Echelard Y and Meade H. Expression of the Antibody hBR96-2 in the Milk of Transgenic Mice and Production of hBR96-2 Transgenic Goats. *Theriogenology* Vol 47, Number 1 214-219 (1997)
  13. Edmunds T, Van Patten SM, Pollock J, Hanson E, Bernasconi R, Higgins E, Manavalan P, Ziomek C, Meade H, McPherson JM, and Cole ES. Transgenically produced human antithrombin: structural and functional comparison to human plasma-derived antithrombin. *Blood*, 91: 4561-71, 1998.
  14. Young MW, Meade H, Curling JM, Ziomek CA, Harvey M. Production of recombinant antibodies in the milk of transgenic animals. *Res Immunol*. 1998 Jul-Aug;149(6):609-10.
  15. Meade HM and Ziomek C. Urine as a substitute for milk? *Nat Biotechnol*, 16: 21-2, 1998
  16. Young MW, Meade HM, Curling JM, Ziomek CA, and Harvey M. Production of recombinant antibodies in the milk of transgenic animals. *Res Immunol*, 149: 609-10, 1998.
  17. Baguisi A, Behboodi E, Melican DT, Pollock JS, Destrempes MM, Cammuso C, Williams JL, Nims SD, Porter CA, Midura P, Palacios MJ, Ayres SL, Denniston RS, Hayes ML, Ziomek CA, Meade HM, Godke RA, Gavin WG, Overstrom EW, and Echelard Y. Production of goats by somatic cell nuclear transfer. *Nat Biotechnol*, 17: 456-61, 1999.
  18. Newton DL, Pollock D, DiTullio P, Echelard Y, Harvey M, Wilburn B, Williams J, Hoogenboom HR, Raus JC, Meade HM, and Rybak SM. Antitransferrin receptor antibody-RNase fusion protein expressed in the mammary gland of transgenic mice. *J Immunol Methods*, 231: 159-67., 1999.
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  21. Echelard Y and Meade HM. Toward a new cash cow. *Nat Biotechnol*, 20: 881-2, 2002.
  22. Stowers AW, Chen Lh LH, Zhang Y, Kennedy MC, Zou L, Lambert L, Rice TJ, Kaslow DC, Saul A, Long CA, Meade HM, and Miller LH. A recombinant vaccine expressed in the milk of transgenic mice protects Aotus monkeys from a lethal challenge with Plasmodium falciparum. *Proc Natl Acad Sci U S A*, 99: 339-44, 2002.
  23. Echelard Y, Meade HM. Protein Production in the milk of transgenic animals. In "Gene Transfer and Expression in Mammalian Cells", edited by S. C. Makrides, New Comprehensive Biochemistry Vol. 38, General Editor: G. Bernardi. Elsevier BV. 2003
- Behboodi E, Memili E, Melican DT, Destrempes MM, Overton SA, Williams JL, Flanagan PA, Butler RE, Liem H, Chen LH, Meade HM, Gavin WG, Echelard Y. Viable transgenic goats derived from skin cells. *Transgenic Res* 13:215-224, 2004.

### **C. Issued Patents**

1. 4,873,316 (1989) Isolation of exogenous recombinant proteins from the milk of transgenic mammals
2. 5,168,049 (1992) Production of streptavidin-like polypeptides
3. 5,272,254 (1993) Production of streptavidin-like polypeptides
4. 5,688,677 (1997) Deoxyribonucleic acids containing inactivated hormone responsive elements
5. 5,750,172 (1998) Transgenic non human mammal milk
6. 5,827,690 (1998) Transgenic production of antibodies in milk
7. 5,843,705 (1998) Transgenically produced antithrombin III
8. 5,849,992 (1998) Transgenic production of antibodies in milk
9. 6,441,145 (2002) Transgenically produced Antithrombin III
10. 6,528,699 (2003) Transgenically produced non-secreted proteins
11. 6,548,653 (2003) Erythropoietin analog-human serum albumin fusion.
12. 6,593,463 (2003) Modified MSP-1 nucleic acid sequences and methods for increasing mRNA levels

### **D. Ongoing Research Support**

**National Institute of Health, National Heart, Lung and Blood Institute.**

Phase I/II Small Business Innovation Research Program. July 2004–December 2006. Title: Improved yield of human albumin from transgenic cow milk. Grant No. 1 R44 HL078300-01. Role on project: Principal Investigator.

The overall goal of this program is to provide an abundant, cost-effective, biosecure source of human albumin for human therapeutic use by producing recombinant human albumin (rhA) in the milk of transgenic cows

## RESEARCH PAPERS

# PRODUCTION OF HUMAN TISSUE PLASMINOGEN ACTIVATOR IN TRANSGENIC MOUSE MILK

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We set out to express an exogenous gene in the mammary epithelium of transgenic mice in the hope that the encoded protein would be secreted into milk. The promoter and upstream regulatory sequences from the murine whey acid protein (WAP) gene were fused to cDNA encoding human tissue plasminogen activator (t-PA) with its endogenous secretion signal sequence. This hybrid gene was injected into mouse embryos, resultant transgenic mice were mated, and milk obtained from lactating females was shown to contain biologically active t-PA. This result establishes the feasibility of secretion into the milk of transgenic animals for production of biologically active heterologous proteins, and may provide a powerful method to produce such proteins on a large scale.

**G**enes injected into mouse embryos may be incorporated into the germ line and be expressed in patterns that mimic those of their endogenous counterparts<sup>1,2</sup>. The pattern of spatial and temporal expression of foreign genes in transgenic animals can be controlled by prior manipulation of the signals regulating gene expression. We introduced into mice a construct designed to express a foreign protein in the lactating mammary epithelium in which 5' sequences from the whey acid protein gene were fused with a cDNA coding for tissue plasminogen activator. We demonstrate here that such an approach is a feasible means of expression of foreign proteins into secreted milk.

Whey acid protein (WAP) is the most abundant whey protein in mouse milk<sup>3</sup>. During lactation, the level of WAP RNA in the mammary gland increases approximately 340-fold from the barely detectable levels present in the mammary gland of virgin mice<sup>4</sup>, and accumulates in lactating tissue at levels of about 15% of the total mRNA<sup>4-6</sup>. Expression of the WAP gene and the stabilization of its mRNA are subject to complex regulation by both steroid and peptide hormones<sup>4</sup>, and putative regulatory protein binding sites within the WAP promoter have been described<sup>7</sup>. Since WAP is found in mouse milk at high levels and the gene had been previously cloned and

characterized<sup>8,9</sup>, we chose to utilize WAP upstream DNA as a promoter in our expression vector. By demonstrating secretion of a foreign protein into milk, the results reported here extend earlier observations showing upstream sequences from the WAP gene were able to target gene expression to the lactating mammary gland in transgenic mice<sup>10</sup>.

### RESULTS AND DISCUSSION

**Construction of t-PA expression vector.** A mammary expression vector was constructed in which 5' sequences from the whey acid protein gene were fused with a cDNA coding for tissue plasminogen activator. t-PA has great potential clinical utility as an agent to dissolve fibrin clots and thus treat victims of myocardial infarction and other life threatening conditions. Its advantage relative to other pharmacological agents such as streptokinase and urokinase lies in its specificity for fibrin. Moreover, assay of its biological activity is both sensitive and convenient and an antibody kit is available for routine screening.

The t-PA gene utilized here was a cDNA clone from a human uterus cDNA library. The t-PA DNA sequence was determined previously and the protein expressed in C127 cells using bovine papilloma vectors<sup>11</sup>. The construct shown in Figure 1 (designated WAP-tPA) is a tripartite fusion consisting of 2.6 kb of upstream DNA from the WAP gene through the endogenous CAP site, t-PA cDNA beginning in the untranslated 5' region, and the polyadenylation/termination signals from SV40. This tPA/SV40 polyadenylation cassette was characterized previously<sup>11</sup>. The secretion signal sequence in this construct derives from the native t-PA gene; the analogous signal encoding region from the WAP gene was removed in the construction. We did not know *a priori* whether the t-PA secretion signal would function efficiently in native mammary epithelial cells. However, since many proteins with different signal peptides are secreted efficiently by mammary cells and since milk proteins can be efficiently transported by membrane systems from other cells<sup>12</sup>, it seemed likely that no specific signal sequence is required for secretion in mammary tissue.

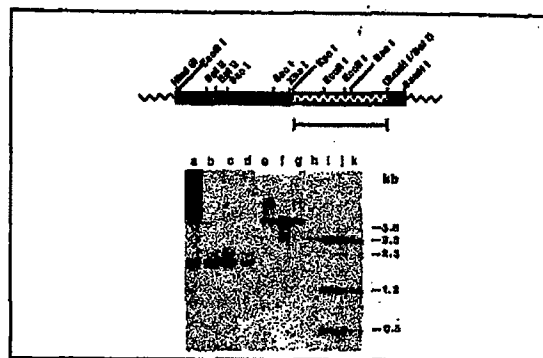
**Transient expression in tissue culture.** To test WAP-tPA for its ability to specify production of secretable t-PA in mammary epithelial cells, the fusion gene was transfected into the mammary cell line, MCF7. Tissue culture supernatants collected 48 hours after transfection were loaded into wells of an assay plate as shown in Figure 2A. The assay consisted of lysis (clearing) of an artificial fibrin clot laid down as a matrix in agarose poured into the wells of a tissue culture plate. The degree of clearing, determined by estimating the diameter of the cleared ring

emanating from the sample loading well, indicates the amount of active t-PA in the sample. In five repetitions from two separate transfections, the level of t-PA secreted into the culture medium was found to be 2.5, 1.5, 10, 5, and 5 ng/ml. The same five samples were also assayed by ELISA using a polyclonal anti-human t-PA antibody. By this assay, the expression levels were either below the detection limit (approximately 2 ng/ml) or 11, 10, and 10 ng/ml, respectively. Thus, whether assayed by biological activity or immunologically, MCF7 cells transfected with WAP-tPA were able to secrete t-PA.

**Generation of transgenic animals.** The plasmid WAP-tPA was injected into one-cell pronuclear mouse embryos as a purified Hind-3/BamHI fragment containing no prokaryotic sequences. The injected embryos were implanted into pseudopregnant females and 29 mice were born. Of these, seven were identified as being transgenic by diagnostic Southern blot hybridization with a human cDNA t-PA probe. Under conditions of high stringency, this probe does not hybridize with the endogenous mouse t-PA gene. The blot patterns of three positive mice, #wt1-26, wt1-25 and wt1-7 are shown in Figure 1. By comparison to the hybridization intensity obtained with positive controls, the number of copies of the injected fragment present in the genomes of these transgenic mice was estimated to be between 20 and 50. Digestion with SacI (lanes b-d) yielded a diagnostic band of 1.75 kb that spans the WAP and t-PA junction and hybridizes to the probe (Fig. 1). The intact plasmid digested with SacI was used as a positive control for this digest (lane a). Exogenous DNA injected into embryos tends to form concatamers even when introduced as a fragment with non-cohesive ends. The 2.3 kb band seen in lanes b-d corresponds to the 3' end of the t-PA gene (which does not contain SacI sites), apparently ligated to the 5' end of the WAP promoter, and through to the first Sac I site in the WAP DNA. The presence and size of this fragment is diagnostic of head-to-tail concatamers.

The EcoRI digest (control, lane h; experimentals, lane i-k) showed the expected 472 bp band internal to the t-PA gene. In addition, a 3.3 kb band can be seen that represents the 5' region of the t-PA gene and extends through the WAP gene to 5' boundary EcoRI site. Thus, despite the fact that the WAP EcoRI site was near the end of the injected fragment, it appeared to be intact in the genomic DNA of this transgenic animal. The 1.2 kb band represents the 3'-most region of the t-PA gene, which must have ligated head-to-tail to the 5' end of the WAP gene, leaving the t-PA gene bounded on its 3' end by an EcoRI site. Interestingly, the weak 2.3 kb band indicates that some of the copies of the fragments formed concatamers in a head-to-head configuration. KpnI digestion (lanes e-g) produced a single band of 4.9 kb, as expected. It is impossible to determine from this Southern blot whether all copies of the concatomer integrated at a single or at multiple sites.

**Expression of biologically active t-PA in milk.** Mice #wt1-26 and wt1-25 were mated to wild type males and had no apparent difficulty in conception or maintenance of pregnancy. Several days after parturition, milk was obtained from the females and was assayed for t-PA activity. Since wt1-11 was a male, it was necessary to obtain transgenic female progeny, mate them, and obtain milk from the second generation females after parturition. We have characterized expression from one progeny animal of this lineage, wt2-102. Since it was not known whether the mouse milk itself would interfere in the fibrin clot assay, we used standards consisting of recombinant t-PA added to milk from nontransgenic mice. As shown in Figure 2b, dilution of standards in milk did not affect the

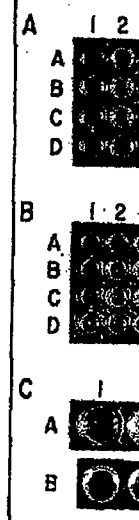


**FIGURE 1** Generation of transgenic animals. Top portion: Restriction map of WAP-tPA; Bottom portion: Southern blot of DNA from tails. Lanes a and h show 500 pg of WAP-tPA DNA digested with SacI or EcoRI, respectively. Lanes b, c, and d contain 5 µg of DNA from mouse wt1-26, wt1-25 and wt1-7, respectively, digested with SacI. Lanes e-g are from mouse tails of wt1-26, wt1-25 and wt1-7, respectively, cut with KpnI, and lanes i-k are these DNAs cut with EcoRI. Lanes a-d were run on a separate gel than the rest of the lanes. Negative control DNAs did not show any hybridization to this probe under these conditions (not shown).

apparent concentration (in comparison to standards diluted in PBS), nor was there background clearing in the negative control sample wells. In this figure it can be seen that milk from wt1-26 cleared the fibrin clot to a significant extent. By comparison with lysis catalyzed by known amounts of added t-PA, the concentration was calculated to be about 200 ng/ml. In parallel assays, milk obtained from wt1-25 and wt2-102 was shown to contain 200 ng/ml and 400 ng/ml of t-PA (data not shown). When plates were incubated longer than 24 hours, minor clearing was seen in control wells containing milk from untransfected mice, but this was always significantly less than clearing seen from milk of any of the transgenic lineages. The origin of the residual fibrinolytic activity in non-transgenic mouse milk is not known. However, the presence of low levels of plasminogen activator (PA) in the lactating mammary gland of rodents<sup>12</sup> raises the possibility that some fibrinolytic protein is present naturally in milk.

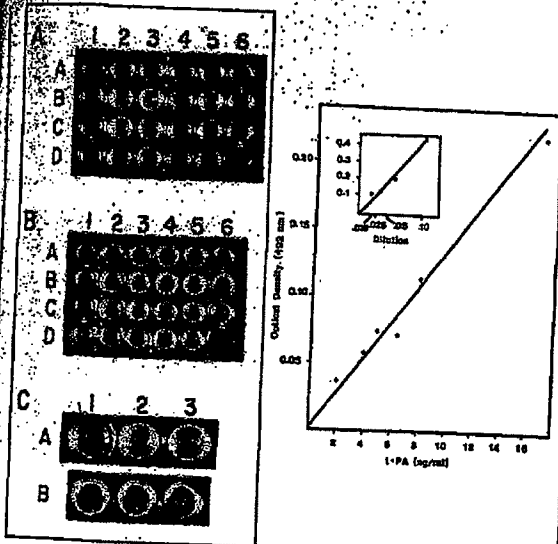
Milk from mice wt1-26, wt1-25 and 2-102 was assayed by ELISA using an anti-human t-PA polyclonal antibody (Fig. 3). A standard curve was generated by addition of known amounts of human t-PA to mouse milk. Identical curves were generated by dilution of t-PA in cow milk and aqueous buffer (not shown). The inset of Figure 3 shows results of an assay of serial dilutions of milk from wt1-26 confirming that about 300 ng/ml of t-PA was present in this sample. Milk from wild type mice showed no signal in the ELISA. Milk from wt1-25 and wt2-102 contained t-PA at concentrations of 114 and 460 ng/ml, respectively (data not shown). The measurements by fibrin clot lysis and ELISA were not sufficiently accurate to determine precisely the specific activity of the t-PA produced in milk. Further studies of the purified protein (now in progress) will establish whether the specific activity of the protein is identical to that produced by melanoma cells and, by recombinant DNA methods. Pilot studies indicate that t-PA remains stable and bioactive in whole milk for at least 48 hours at 37°C, and can be stored at -80°C (data not shown).

Since WAP RNA constitutes as much as 15% of the poly A (+) mRNA in the lactating mammary gland, it is probable that the level of t-PA in the milk of these mice is far below the level of endogenous whey acid protein. This



**FIGURE 2** Clot lysis assay. Row A, column 1 shows expression of t-PA in milk from transgenic mice (wt1-26, wt1-25, wt2-102) at 20, 10, and 5 ng/ml. Row B, column 1 shows expression of t-PA in milk from transgenic mice (wt1-26, wt1-25, wt2-102) at 20, 10, and 5 ng/ml. Row C, column 1 shows expression of t-PA in milk from transgenic mice (wt1-26, wt1-25, wt2-102) at 20, 10, and 5 ng/ml. Row D, column 1 shows expression of t-PA in milk from transgenic mice (wt1-26, wt1-25, wt2-102) at 20, 10, and 5 ng/ml. Row A and B show expression of t-PA in milk from transgenic mice (wt1-26, wt1-25, wt2-102) at 20, 10, and 5 ng/ml. Row C and D show expression of t-PA in milk from transgenic mice (wt1-26, wt1-25, wt2-102) at 20, 10, and 5 ng/ml.

**FIGURE 3** Quantitation of t-PA in milk. Row A shows expression of t-PA in milk from transgenic mice (wt1-26, wt1-25, wt2-102) at 20, 10, and 5 ng/ml. Row B shows expression of t-PA in milk from transgenic mice (wt1-26, wt1-25, wt2-102) at 20, 10, and 5 ng/ml. Row C shows expression of t-PA in milk from transgenic mice (wt1-26, wt1-25, wt2-102) at 20, 10, and 5 ng/ml. Row D shows expression of t-PA in milk from transgenic mice (wt1-26, wt1-25, wt2-102) at 20, 10, and 5 ng/ml.



**FIGURE 2** Clot lysis bioassay of secreted t-PA. (A) Transient expression of WAP t-PA in MCF7 cells. Two transfections were done, #1 (two repetitions, a and b) and #2 (3 repetitions, a, b, and c). In row A, columns 1 through 6 are recombinant DNA t-PA standards diluted in culture medium at concentrations of 20, 10, 5, 2.5, 1.25, and 0 ng/ml. Row B contains samples from transfection #1. In columns 1, 2, and 3 are three dilutions of sample from one transfection: 1×, 5×, and 25×, respectively. In columns 4, 5, and 6 or row B are similar dilutions from the repeat transfection. Row C, columns 1–3, row C, columns 4–6, and row D, columns 1–3 contain similar dilutions from the three repetitions of transfection #2. (B) Milk from transgenic mouse #wt1–26. Following identification of mouse #wt1–26 as a positive transgenic, the mouse was mated to a wild type male. Seventeen days after the first litter was born, milk was removed from the lactating female following stimulation with oxytocin. Milk was diluted in PBS by 50% and stored frozen. Milk was diluted further in PBS as indicated below just prior to assay and added to the wells of a fibrinogen spot assay plate. The positive controls were generated by addition of recombinant t-PA to media composed of either 10% negative mouse milk (row A), 10% negative cow milk (row B), or PBS (row C). Concentrations of t-PA in the milk dilution curves, from columns 1 through 5 are: 40, 20, 10, 5, and 0 ng/ml. The concentrations in the PBS dilution curve, from column 1 through 6 are: 40, 20, 10, 5, 2.5, and 0 ng/ml. In row A, column 6 is the milk from mouse #wt1–26 at a final concentration of 10% and in row B, column 6 is the milk at a concentration of 5%. This photograph was taken after approximately 8 hours of assay incubation time. The negative mouse milk used for these controls was pooled from outbred (CD-1) mice in different stages of lactation. In other negative controls (not shown) milk was used from inbred females of the same strain used for microinjection and was obtained at the same stage of lactation as the positive sample. The specificity of recombinant t-PA secreted into mouse milk was shown to be plasminogen in other experiments (not shown) in which plasminogen was omitted from the agarose matrix in similar fibrinogen spot assays. (C) Enlargement of key data of sections A and B. Row A shows an enlargement of the data of section A (above), row C, columns 1–3; Row B shows one of the data of section B (above), row A, columns 4–6.

**FIGURE 3** Quantitation by ELISA of recombinant t-PA secreted into milk of mouse wt1–26. The standard curve was performed in negative mouse milk diluted to a final concentration of 10% with PBS, to which was added t-PA supplied with the kit as indicated. The inset shows milk from mouse #wt1–26 in dilutions as indicated. The dilution of .1 refers to a final concentration of 10% milk. In each dilution of transgenic milk, samples were supplemented with negative mouse milk in order to keep the final concentration at 10%. All points of the control curve and the experimental (inset) curve have the background value (the value determined for negative mouse milk) subtracted.

could be due to many factors. Preliminary data indicate that variation in t-PA expression levels among transgenic mice containing WAP-tPA may be considerable, suggesting that the chromosomal integration site may play a key role in establishing levels of expression from this construction. In fact, one transgenic mouse (not shown) appeared to express virtually no t-PA in the milk. Thus, analysis of additional animals may identify those which produce more t-PA. In addition, intragenic and/or noncoding 5' and 3' sequences from the WAP gene, missing from the construction introduced into mice in these experiments, may play important roles in RNA stability. Considerable work remains to be done to configure the t-PA expression vector for maximal expression.

We demonstrate here that a foreign protein, human tissue plasminogen activator, can be secreted into the milk of transgenic mice under the control of a mammary-specific promoter. Thus, concerns that foreign proteins produced in the mammary gland might not be secreted, accurately processed, or be sufficiently stable in milk appear to be reduced by these results. The advantages of producing foreign proteins in this manner include the fact that milk is well characterized biochemically and that many of the genes encoding key milk proteins have been cloned. In addition, many milk-specific genes are expressed in the lactating mammary gland at high levels under hormonal control and in a tissue-specific manner. Thus, with expression cassettes similar to the one described here, it should be possible to target precisely foreign gene expression to the lactating mammary epithelium. Factor IX and t-PA have been produced in the blood of transgenic mice<sup>14,15</sup>; the ability to produce these proteins in milk would facilitate their collection. The ultimate goal of our experiments is to express foreign proteins in the milk of farm animals. Since production of transgenic farm animals has been achieved<sup>16</sup>, this presents a reasonable possibility. Although many technical hurdles remain, the data presented here demonstrate that transgenic animals may become an attractive alternative for future production of genetically engineered biologically active proteins.

#### EXPERIMENTAL PROTOCOL

**Construction of expression vector.** A Hind-III site was added at the 5' end of the 2.6 kb WAP promoter sequence (Ref. 5) by digestion at the single EcoRI site in the WAP promoter, filling in with Klenow and dNTPs, and ligation of Hind-3 linkers, all by standard protocols. The t-PA cDNA (Ref. 11)-SV40 termination/polyadenylation cassette (see Ref. 11) was inserted into the polylinker region of the WAP promoter vector as a KpnI-Bam HI fragment.

**Generation and analysis of transgenic animals.** To purify the eukaryotic sequences for microinjection, WAP-tPA was digested with Hind-3 and BamHI, the fragments separated by gel electrophoresis, and the 4.9 kb fragment purified by binding to glass filter fiber papers<sup>17</sup>. After elution and concentration by ethanol precipitation, the DNA was suspended for microinjection in 10mM Tris, .05 mM EDTA, pH 7.5 at a concentration of 5 ng/microliter. The regulatory/coding restriction fragment from pWAP-tPA was microinjected into one cell fertilized embryos as described previously<sup>18</sup>. At four weeks of age, tail sections were taken from mice born from these injections, digested with proteinase K, phenol-chloroform extracted, then digested with various restriction enzymes. DNA was electrophoresed on a Tris-borate gel, blotted to nitrocellulose, and hybridized with a probe consisting of the entire coding region of t-PA cDNA (see bold line under the restriction map of Fig. 1). Labeling was done by extension of random hexamers to a specific activity of  $1 \times 10^5$  cpm/ $\mu$ g.

**Calcium phosphate transformation.** MCF7 cells were plated in 100 mm dishes at densities of  $5 \times 10^6$  or more per dish at least one day prior to transfection. Transfections were performed as described previously<sup>19</sup> and transient supernatants collected 48 hours after transfection were assayed for t-PA.

**Fibrin clot lysis assay.** The fibrin clot assay measures the ability of t-PA to digest fibrinogen matrices which are laid down

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in a background of agarose, thrombin and plasminogen within the wells of a plate <sup>20</sup>. A small hole is bored through the agarose substrate upon hardening and 25 microliters of the samples are loaded into each of the holes. As t-PA diffuses into the agarose, clearing of the fibrinogen is evident visually and the amount of clearing is directly proportional to the amount of active t-PA. These assays are extremely sensitive and reproducible.

**ELISA assay.** Assays were performed with the IMUBIND ELISA kit produced by American Diagnostica Inc. The assay is a double antibody sandwich in which the primary antibody is a goat antiserum raised against t-PA from human uterus and the second antibody is a peroxidase conjugated anti-t-PA IgG. The standard curves were performed in negative mouse milk diluted to a final concentration of 10% with PBS, to which was added t-PA supplied with the kit.

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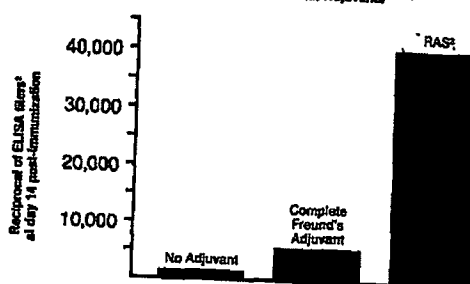
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## Transgenic rabbits for the production of biologically-active recombinant proteins in the milk

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### Abstract

The use of live bioreactors for the expression of human genes in the mammary gland of transgenic animals is one of the most cost-effective ways for the production of valuable recombinant therapeutic proteins. Among the transgenic species used so far, rabbits are good candidates for the expression of tens to hundreds of grams of complex proteins in the milk during lactation. The lactating mammary gland of rabbits has proven to be effective in the processing of complex proteins. In this work, the potential use of rabbits as bioreactors is discussed based on our results and the published data. © 1999 Published by Elsevier Science B.V. All rights reserved.

**Keywords:** Caseins; Mammary gland; Milk genes; Transgenic rabbits; WAP

### 1. Introduction

Since the first transgenic farm animals were generated in 1985 [1], relatively little time has elapsed; however, important achievements have been made in the transgenic technology. Among those are: the creation of knock out mice [2]; pigs for the production of organs intended for xenotransplantation to humans [3]; farm animal species secreting complex drugs in their milk [4]; and more recently the birth of transgenic cloned ruminants from differentiated adult or fetal cells through nuclear transfer.

Among the areas in which the transgenic technology is expected to exert a powerful influence, the expression of recombinant protein genes in the milk of transgenic livestock is undoubtedly one of the most developed at present. In 1987 Gordon and coworkers showed for the first time that transgenic mice could appropriately process a complex human protein gene like the tissue plasminogen activator gene [5]. At present, hundreds of lines of transgenic mice expressing recombinant proteins in their milk have been produced. The mice have served only as a predictive model for the genera-

tion of transgenic farm animals, however the choice criteria for selecting the most suitable species for gene farming is usually based on the quantity of protein needed per year. A simplified rule is: the production of a protein in tons should be carried out by cows, in hundreds of kg by sheep or goats, and in kilograms per year by rabbits. Transgenic rabbits fit well as an intermediate animal for the production of limited amount of proteins [6]. Rabbits have short reproductive interval, can be reared under specific pathogen-free conditions and their milk is relatively well characterized [7]. A transgenic lactating doe can yield up to 10 kg of milk per year under semi-automatic hygienic milking condition [8]. All these features make rabbits attractive for the mammary-gland specific expression of recombinant proteins [9,10].

### 2. Factors affecting the efficiency of generation of transgenic rabbits

The success of gene transfer in rabbits can be expressed as: (1) the survival rate (animals born/injected embryos transferred); (2) the transgene integration frequency (transgenics/no. of offspring); and (3) the overall efficiency (transgenics/injected embryos). The values

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of these figures vary between gene constructs and the average success rates obtained in rabbits are 10–15% survival rate, 8–12% integration frequency, and 1–2% efficiency [6]. It is important to point out that gene constructs encoding products with possible deleterious side effects and/or with regulatory elements allowing expression during embryogenesis, will cause decreased gene-transfer efficiency because they counterselect the transgenesis as seems to be the case for hEPO gene constructs in transgenic rabbits [11,12].

There are several factors affecting the efficiency of gene transfer in rabbits. They can be grouped in two: factors dealing with the reproductive performance of the rabbits and the state of the art of the embryo micromanipulation techniques, and factors intrinsic to the transgenic technology. To the first group of factors belong the number of ovulations achieved with each superovulation treatment, the age and experience in fostering of the recipient mothers, the type of embryo transfer procedure (surgical vs. laparoscopic), the breed of the rabbits, nutritional and habitat conditions among others. The impact of these factors have been the subject of a recent review article by Brem et al. [6] and although they are of crucial importance, are more susceptible to be improved by means of the human control of each of them.

The second group of factors dealing with the intrinsic nature of the transgenic technology is more complicated to control due mainly to the lack of knowledge that we currently have about them. Among those factors the most important are: lack of control over the integration phenomenon, mosaicism of integration and expression of the transgene, lack of cellular models to predict the behavior of the transgenes, inaccurate regulation of transgene expression, and unknown capabilities of the mammary gland to perform some posttranslational modifications required for the activity of the recombinant protein.

One way to surmount the disadvantages of pronuclear gene microinjection in rabbits could be the establishment of embryonic stem cell lines amenable to be targeted *in vitro* and still be able to contribute to germ-line chimeras after their injection into blastocysts. Although several attempts have been made in this regard, little success have been obtained so far. Embryonic cells derived from the inner cell mass of pre-implantation rabbit embryos have been characterized morphologically and biochemically. ES-like cells cultured for several passages showed low degree of chimerism *in vivo* [13,14]. In other experiment, *in vitro* cultured PGCs were used to produce chimeric rabbits by injecting them into pre-implantation embryos [15]. The chimerism level was also low, although in some animals it was possible to detect it in gonads. Nuclear transfer experiments with ES-like cells from rabbits resulted in normal development until the blastocyst

stage [16], however, no germ line chimerism could be detected. In conclusion, there is not at present any demonstration of germ-line forming chimeras derived from ES-like cells in rabbits. The availability of embryonic stem cells in rabbits would be of great impact for site-specific additive or subtractive gene transfer.

More recently, cloned lambs, cattle, and mice were obtained by the transfer of nuclei isolated from differentiated cells into enucleated oocytes [17–22]. At present, there is no published report about cloned rabbits born after somatic nuclear transfer. *In vitro*-derived blastocysts after fusion of either mammary epithelial cells, or fetal fibroblast cells with nucleated rabbits oocytes were reported by [23] and also by our group [24]. Obviously, there is still much to be carried out before somatic nuclear transfer becomes the technique of choice for manipulating the genomic content of the rabbit. However, the availability of cell lineages capable of participating in embryonic development is a prerequisite for altering the genome by homologous recombination (gene targeting) and also offers new possibilities in additive gene-transfer experiments. On the other hand, the use of somatic cloning in rabbits will be probably limited if compared with the potential applications of this methodology in ruminants for instances. However, tempting applications such as identical clones of rabbits useful for testing human drugs with a homogenous physiological response would be of great value for the pharmaceutical industry.

Mosaicism of integration and expression of the transgenes is very common in rabbits. It is worth to notice that almost 30% of the founder transgenic animals are mosaics [25]. This frequency is very similar in rabbits as in mice [6]. According to the data of Brem and coworkers, approximately 10 and 20% of founders will not transmit the transgene to their offspring. Mosaicism is already detected in *in vitro* cultured embryos after the microinjection of one-cell embryos with a lacZ reporter gene. In our hands about 40% of the embryos expressed the transgene only in one or a few blastomeres [26]. This phenomenon could be partially a result of the fact that embryos might still contain episomal gene constructs, but this suggests also a high degree of integration mosaicism.

### 3. The need of cellular models

One of the main hurdles of current mammary gland transgenesis is the lack of appropriate cellular models for testing transgene constructs before spending time and effort to generate transgenic mice or farm animals. Mice have acted as laboratory models for testing gene constructs, however, as shown by multiple reports [12,27] the predictive potential of the transgenic mice is very limited. Therefore, cell models, although are not a

mirror of physiological processes of whole animals, are of outstanding interest for fast testing of transgenes. One of the main advantages of the cell systems is that one can test several different gene constructs in a short period of time at affordable costs. To date HC-11 cells are the more used cell models for mammary gland transgenesis. This cell line [28] is a clonal population isolated from the COMMA-1D mouse mammary cells [29]. The cells displayed a normal phenotype and the endogenous  $\beta$ -casein milk can be rapidly induced in vitro without the requirement of an extracellular matrix formation or co-cultivation with other cell lines [30].

Although HC-11 cells can be transfected with heterologous gene constructs, there is no doubt that the establishment of homologous (rabbit) epithelial cell lines could be of great value for the study of transgenes to be expressed in this species. For that reason, we developed a cell culture of mammary epithelial cells, from day-30 pregnant albino New Zealand White doe. This cell culture have not been transformed, however, after more than 30 passages in vitro it keeps the characteristics of the original isolate consisting probably of a mixture of epithelial and mioepithelial cells (Fig. 1). This cell isolate was termed FY-2.

FY-2 cells have been used in our laboratory for the transient expression of reporter genes located under the control of mammary-gland specific promoters. As it is shown in Fig. 2, expression of luciferase from a bovine  $\beta$  casein promoter was induced in FY-2 cells in the presence of prolactin, insulin and dexamethasone. The

inclusion of the prolactin receptor gene in the transfection cocktail further enhanced transcription from the  $\beta$ -casein promoter. Similarly, we have tested a set of murine whey acidic protein (WAP)-hGH construct in these cells with high transfection efficiency, and with secretion of the reporter protein gene to the culture medium (not shown).

In the FY-2 cells there is a notable increase of nuclear Stat5 after induction with the lactogenic hormones as indicated by Western blot assays using monoclonal antibodies against mouse Stat5a,b (Fig. 3 upper panel, lanes 4–6). There was also a modest induction of Stat5 in cytoplasmic extracts of FY-2 cells coincident with the induction pattern observed in the nuclear extract (Fig. 3 lower panel, lanes 4–6). It was interesting to find in both nuclear and cytoplasmic extracts the presence of Stat5 in the proliferating cells (Fig. 3, lane 1, both panels). This phenomenon does not occur in mouse epithelial HC-11 cell line, and probably reflects the non-clonal mixed nature of the FY-2 isolate. In gel retardation assay experiments, it was shown the binding of a  $\beta$  casein labeled oligonucleotide to proteins in the whole cell extract of cells induced only with prolactin (Fig. 4, lanes 2–4). Binding was stimulated further when cells were induced with all three lactogenic hormones (lanes 5–7). There was no binding in the proliferating cells or after retrieval of the induction hormones (lanes 1 and 8–9, respectively).

In summary all this data indicate that in FY-2 rabbit cells, a mechanism similar to that discovered in mice is



Fig. 1. Microphotograph of rabbit cell isolate FY-2 after double immunostaining with monoclonal antibodies against rabbit  $\alpha$  and  $\gamma$  actin (asterisks) and keratins 10,17,18 and 19 (arrow heads). Cells were cultured for three days in DMEM:F12 medium (Hyclone, Logan UT, USA) supplemented with insulin, EGF, pyruvate, glutamine, antibiotics and 10% fetal calf serum). At hyperconfluency cells were immunostained using double staining enhancing kit (Histostain-DS) and monoclonal antibodies from Zymed (Zymed Laboratories, San Francisco, CA, USA). The procedure was performed following manufacturer's instructions. After the addition of the primary antibodies, reactions were developed with secondary antibodies conjugated with streptavidin-alkaline phosphatase (anti-actin; gray color in the picture, indicates cells of muscular origin) or horse-radish peroxidase (anti-keratins; dark black, indicates cells of epithelial origin). Magnification 100  $\times$ .

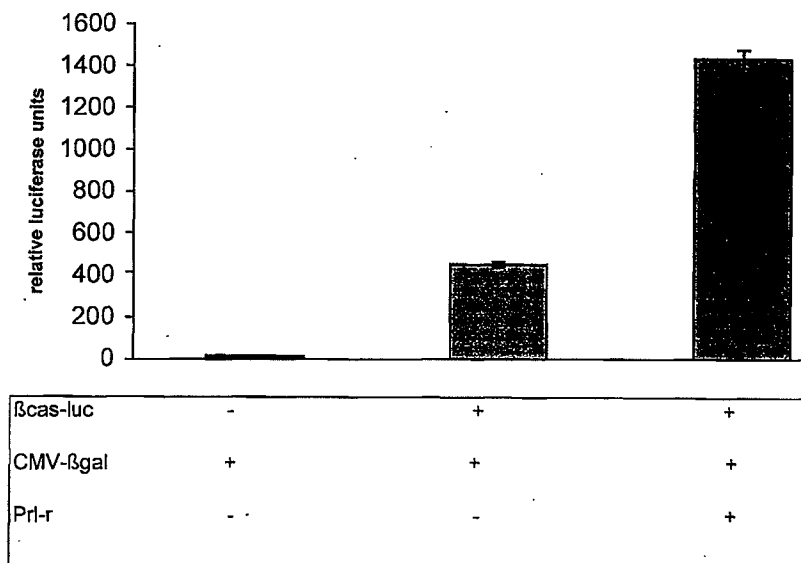


Fig. 2. Luciferase activity after transfection of FY-2 cells with  $\beta$ -casein-luciferase reporter gene and hormonal induction. FY-2 cells were transfected with a combination of the reporter plasmid bovine  $\beta$ -casein-luciferase (5  $\mu$ g), the CMVB-gal plasmid (5  $\mu$ g) and the prolactin receptor gene (40 ng) as indicated. Transfected cells were treated for 14 h with 1  $\mu$ g/ml ovine prolactin, 5  $\mu$ g/ml insulin and  $10^{-7}$  M dexamethasone. Cellular extracts were prepared and luciferase and  $\beta$ -gal activities were determined in the same Berthold luminometer. Luciferase activity was normalized to  $\beta$ -gal measurements. All the experiments were repeated at least three times.

active for prolactin-induced mammary gland gene transcription. Current experiments are in progress in order to clone the epithelial subpopulation present in the FY-2 cell isolate and to deeply study the induction pattern of a endogenous  $\beta$ -casein milk protein gene in these cells. The availability of such a cell line should help in the understanding of the mechanisms underlying regulation of mammary gland gene expression and also for testing gene constructs intended for transgenic rabbit programs.

The other two factors greatly affecting the efficient production of recombinant proteins in the milk of transgenic rabbits, i.e. the inaccurate regulation of transgene expression, and the limited capability of the mammary gland to perform certain posttranslational modifications of the recombinant protein will be discussed within the next section by using examples from published data.

#### 4. Production of proteins of high value by transgenic rabbits

The possibility of establishing transgenic mice and farm animals has opened new ways for the production of pharmaceutical proteins. The advantages of this method are that the production of proteins *in vivo* is more accurate and efficient than *in vitro*. The arising costs are 5–10 times lower than in tissue culture. The mammary gland is the most interesting organ for the production of recombinant proteins (for reviews see

refs. [31–34]. It has an enormous physiological potential for the daily production of proteins under highly hygienic standards.

#### 5. Expression in the mammary gland

In rabbits, caseins are the major protein constituents of milk and their concentration in rabbit's milk is above 60 mg/ml [35]. In the whey fraction, the WAP is abundantly represented and might account for at least 15% of the total milk mRNA. WAP is expressed at 1.5 mg/ml in the milk of the rabbits [35]. The promoters of all these major milk protein genes have been cloned, studied, and used for the expression of transgenes in the mammary gland of mice and rabbits (Table 1; for a review see refs. [36,37]).

#### 6. Gene constructs controlled by whey promoters

##### 6.1. WAP promoter

Rabbit and mouse WAP-controlling sequences have been extensively used for the generation of transgenic mice and rabbits. Despite giving high expression levels of transgenes in the mammary gland in most of the cases studied so far, the WAP promoter also have elicited ectopic expression in the transgenic animals. In one study, murine WAP was expressed ectopically in transgenic sheep [38]. In general WAP-derived transge-

nes have shown erratic behavior that includes precocious transgene expression, lower levels of expression with regard to the endogenous WAP gene as well as ectopic expression [38,39]. These evidences point to the fact that WAP sequences might not be the regulatory element of choice when a tightly-regulated mammary gland expression is desired. Especially when ectopic expression of a given transgene is expected to cause deleterious side effects. An alternative to designing expression cassettes with stricter mammary-gland specificity is to produce the desired protein as inactive or less active form.

Brem and coworkers [40] produced several transgenic rabbit lines expressing up to 4 g/l of the human growth hormone gene (hGH) under the control of a 2.4 kb murine WAP promoter. Although there was no hGH detectable in the blood serum of transgenic rabbits and all the animals were healthy, fertile, and without changes in growth parameters, the same gene construct in mice, resulted in high-level expression of hGH in the brains [41]. Authors suggested that this new phenotype could be attributed to a combination of the WAP promoter and the hGH structural gene.

In our group we generated five transgenic rabbits with a similar gene construct comprising 2.6 kb of murine WAP regulatory sequences and the hGH gene. The expression levels in the milk were found to be around 50 mg/l. Ectopic transgenic expression was detected in blood serum and ovaries. However, the transgene expression in the rabbit line showed no detrimental effect [42].

More recently, we cloned and used a 6 kb rabbit WAP promoter fragment for the generation of transgenic rabbit lines expressing several human proteins including erythropoietin (EPO) in its cDNA or genomic variants, and humanized chimeric antibodies against human cluster of differentiation 6 (CD6). The hEPO expressing transgenic rabbits have been published else-

where [11,39,43,44]. As a result of these transgenic experiments we generated seven transgenic rabbit female founders with expression levels ranging from 0 to 0.8 ng/ml of hEPO in the milk [43,44]. Despite the low levels of the transgenic hEPO in the milk, it was biologically active as judged by sensitive *in vitro* and *in vivo* assays in hypoxic mice [11,43]. hEPO was ectopically expressed in the ovaries of one founder female transgenic for the genomic variant of hEPO. Serum levels of the hEPO were undetectable and no deleterious effects on the transgenic rabbits were scored [11]. Recently, it was reported that a rabbit WAP promoter sequences fused to the hEPO cDNA resulted in one transgenic rabbit line with ectopic expression leading to high amounts of red blood cells even in males. The transgenic rabbits were unable to reproduce and died prematurely. These experiments suggest that transgenic animals obtained with gene constructs that do not contain insulators should not be used as living fermenters to produce human EPO in their milk at an industrial scale [12].

Antibodies are complex molecules usually produced by lymphocytes in response to pathogenic attacks. However, mouse/human chimeric antibodies against human CD-6, had been achieved in the milk of transgenic mice under the control of rabbit WAP promoter sequences. The secreted antibodies were correctly assembled and biologically active in immunofluorescence assays with CD-6 expressing human peripheral blood lymphocytes (PBL, [45]).

The same antibody gene construct was introduced into rabbits. In a set of experiments, 1007 one cell embryos were microinjected, resulting in 82 pups out of 716 transferred embryos in 38 recipient females. Seven founder animals were produced. The overall efficiency of transgenesis was 0.7%, the pregnancy rate 50%, the survival rate 11.45% and the integration frequency 8.5%. All these figures are in concordance with the

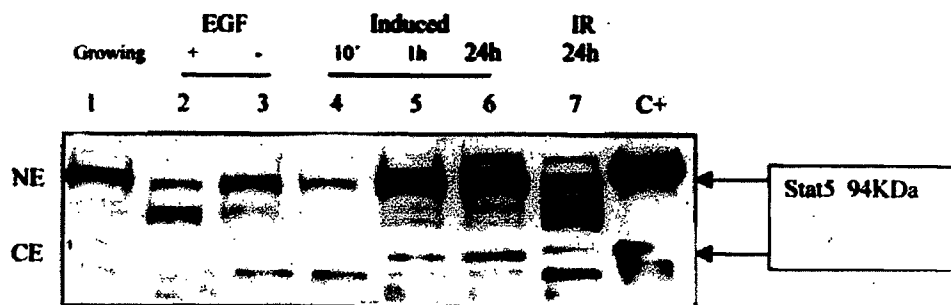


Fig. 3. Stat5 is involved in the prolactin response to lactogenic hormones in FY-2 cells. Western blot analysis of nuclear (upper panel) or cytoplasmic (lower panel) extracts of growing cells (lane 1) hyperconfluent cells in the presence (lane 2) or absence of EGF (lane 3), induced (lanes 4 to 6) or induction retrieved cells (lane 7). As positive control we used purified mouse Stat5 expressed in baculovirus (kindly given by Ch. Beisenhaus, Klinik für Tumorbologie, Freiburg, Germany). Monoclonal antibodies anti Stat5 a,b (Transduction Laboratories, San Juan, CA, USA) were diluted 1:250 and incubated for 1 h at 37°C. Reaction was revealed with peroxidase labelled anti-mouse antibodies (Amersham, Buckinghamshire, UK). The molecular weight of Stat5a is indicated to the right (arrow).

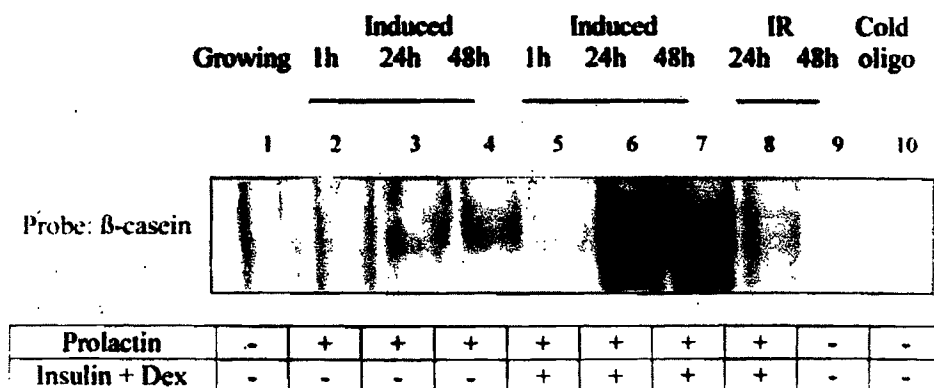


Fig. 4. Binding of  $\beta$ -casein oligos to whole cell extracts of FY-2 cells. Whole cell extracts from FY-2 cells growing (line 1), induced with prolactin alone (lanes 2–4) or with the combination of the lactogenic hormones (lanes 5–7) and retrieved from induction (lanes 8 and 9) were incubated with  $^{32}$ P-labeled alpha dATP bovine  $\beta$ -casein oligonucleotides, and subjected to bandshift assay. In line 10 was run a control of whole cell extract incubated with unlabeled  $\beta$ -casein oligonucleotides.

common accepted efficiency indicators in rabbits. The expression level of the assembled antibodies was 60 mg/l, in the higher expressing line and it was confined to the days 8–15 of lactation. We cannot rule out the possibility that the lack of expression during the remaining days of lactation was a result of the sensitivity of the ELISA test used. This expression level was lower than that achieved in transgenic mice (400 mg/l). The secreted antibody molecules showed specific affinity for its ligand in vitro in flow cytometry analysis with HPB MLT or PBL cells (Fig. 5) and were detected in Western blot assays (data not shown). From this experiments it was clear that the predictive value of transgenic mice should be analyzed with caution.

## 7. Other whey gene promoters

Regulatory elements from other whey genes have been employed in transgenic rabbits. In line with the efforts to express hEPO in the milk of transgenic animals, bovine  $\beta$ -lactoglobulin-hEPO fusion protein was expressed in rabbit's milk under the control of bovine  $\beta$ -lactoglobulin promoter fragment. Expression levels were higher than in all the above discussed reports, but were still low (0.5 mg/ml). The fusion construct included a cleavage site for bacterial IgA protease. Although expression was mainly mammary-gland specific, ectopic production of hEPO was detected in the kidney. The circulating fusion protein was only 15% as active as the recombinant hEPO produced in CHO cells, despite this fact; the fusion construct was not sufficiently safe to be use in large scale production of hEPO [46], and it was still able to elicit polycythemia in lactating rabbits. After protease cleavage, full biological activity was recovered in vitro. The transgenic hEPO was differentially glycosylated with regard to the CHO-derived hEPO, whether this fact is specific for hEPO should be

tested. As it will be discussed below, human extracellular superoxide dismutase was correctly glycosylated in the milk of transgenic rabbits [47].

In another report with ovine  $\beta$ -lactoglobulin controlling sequences, salmon calcitonin (sCT) peptide was expressed as a fusion protein with human alpha lactalbumin in the milk of transgenic rabbits. The fusion construct included a 4.3 kb promoter fragment of the ovine  $\beta$ -lactoglobulin gene. Three founder animals were obtained with expression levels between 1.0 and 2.0 mg/ml. C-terminal amidation in vivo was achieved by extending the sCT by a single glycine residue that provides a substrate for endogenous amidating activity in the mammary gland. As a salient feature of this experiment, 65–90% of amidation was performed by the mammary gland of the lactating females. The sCT peptide was biologically active after enzymatic cleavage and showed no deleterious effect on the circulating calcium levels in vivo in the transgenic animals [48]. This is the first experimental evidence of amidation of a recombinant protein expressed in the mammary gland of transgenic animals. Interestingly, the fused human alpha-lactalbumin was preferentially phosphorylated in the mammary gland of the transgenic rabbits but not in transgenic cattle expressing the protein at 2.4 g/l. The later could indicate that the kinases in the rabbit mammary gland are more active than in other species such as cattle.

## 8. Gene constructs controlled by casein promoters

### 8.1. $\alpha$ S<sub>1</sub>-casein promoter

$\alpha$ S<sub>1</sub>-Casein is present at high concentration in the milk of most mammals, including rabbits [49]. For this reason its regulatory sequences have been extensively used to target the expression of recombinant genes in

Table 1

Summary of published reports on transgenic rabbits expressing foreign proteins in milk<sup>a</sup>

| Promoter element (length in kb)      | Coding region      | Highest expression levels (g/l) | Ectopic expression  | Reference number |
|--------------------------------------|--------------------|---------------------------------|---------------------|------------------|
| mWAP (2.4)                           | hGH (g)            | 4.0                             | Brain               | 40               |
| mWAP (2.6)                           | hGH (g)            | 0.05                            | Blood, ovaries      | 42               |
| mWAP (2.3)                           | EcSOD cDNA         | 3.0                             | Ovaries             | 47               |
| rabWAP (6.0)                         | hEPO cDNA          | $3 \times 10^{-7}$              | Not detected        | 43,44            |
| rabWAP (6.0)                         | hEPO (g)           | $1 \times 10^{-7}$              | Not detected        | 11,39            |
| rabWAP (6.3)                         | hEPO cDNA          | 0.05                            | Blood, other organs | 12               |
| rabWAP (6.0)                         | IORT1 (g)          | 0.03                            | Not detected        | This work        |
| rab $\beta$ -cas (2.0)               | hIL-2 (g)          | 0.34                            | Not detected        | 56               |
| b $\alpha$ S <sub>1</sub> -cas (1.6) | htPA cDNA          | 0.05                            | Not reported        | 50               |
| b $\alpha$ S <sub>1</sub> -cas*      | hIGF-1             | 2.0                             | Not detected        | 54,55            |
| b $\alpha$ S <sub>1</sub> -cas*      | bchymos (g)        | 2.0                             | Not reported        | 52               |
| b $\alpha$ S <sub>1</sub> -cas*      | hNGF cDNA          | 0.2                             | Not reported        | 53               |
| bov $\beta$ LG (2.8)                 | b $\beta$ LG-hEPO  | 0.5                             | Kidney (low)        | 46               |
| ov $\beta$ LG (4.3)                  | h $\alpha$ lac-sCT | 2.0                             | Not reported        | 48               |

<sup>a</sup> Abbreviations: mWAP, mouse WAP; rabWAP, rabbit WAP; b $\alpha$ S<sub>1</sub>-cas, bovine  $\alpha$ S<sub>1</sub> casein; bov $\beta$ LG, bovine betalactoglobulin; ov $\beta$ LG, ovine betalactoglobulin; hGH, human growth hormone; EcSOD, extracellular superoxide dismutase; hEPO, human erythropoietin; g, genomic; IORT1 monoclonal antibody against human CD6; htPA, human tissue plasminogen activator; hIGF-1, human insulin-like growth factor-1; bchymos, bovine chymosin; hNGF, human nerve growth factor; h $\alpha$ lac, human alpha lactoalbumin; sCT, salmon calcitonin.

\* Authors used variable length of promoter fragments ranging from 2.9 to 11.0 kb.

the mammary gland of transgenic animals. We generated transgenic mice and one transgenic rabbit founder from an expression cassette composed of a 1.6 kb of the bovine  $\alpha$ S<sub>1</sub>-casein promoter fragment fused to cDNA encoding the htPA [50]. The expression levels were in the order of 50 mg/l in both species, thus indicating that longer promoter fragments are required for tissue-specific high-level expression in the mammary gland.

The group of Gottfried Brem [6] has generated most of the results using bovine  $\alpha$ S<sub>1</sub>-casein promoter sequences in rabbits. In these experiments, the mammary-gland specific expression cassette consists of bovine  $\alpha$ S<sub>1</sub>-casein sequences providing the 5'-promoter elements and the 3'-elements necessary for mRNA processing. In addition the construct includes intron/exon boundaries known to enhance transgene expression and the signal peptide from the  $\alpha$ S<sub>1</sub>-casein gene [51].

Sixteen lines of transgenic rabbits expressing different variants of the bovine prochymosin gene were created [52]. The expression levels of chymosin varied from 0.5 to 2 g/l in nine lines studied. In all cases, the protein was secreted as an inactive precursor that was readily activated by lowering the pH (pH = 2.5 for 1.5 h) and subsequently neutralized. In the biological activity test, cow milk was clotted at high efficiency with the transgenically produced chymosin.

In another set of experiments, recombinant human  $\beta$ -NGF was placed in the bovine  $\alpha$ S<sub>1</sub>-casein expression cassette. A total of seven lactating transgenic females analyzed so far yielded between 0.1 and 0.2 g/l of the  $\beta$ -NGF. No deleterious effects were found in the transgenic rabbits [53]. The biological activity of such NGF is being studied at present [6].

Eight hIGF-1 expressing lines of transgenic rabbits were established from two  $\alpha$ S<sub>1</sub>-casein expression cassettes comprising 5'-regulatory sequences of 2.9 and 11.0 kb, respectively [54]. Transgene expression was confined to the mammary gland during lactation. Biologically-active hIGF-1 at levels ranging from 0.5 to 2 g/l have been purified from the transgenic milk [55].

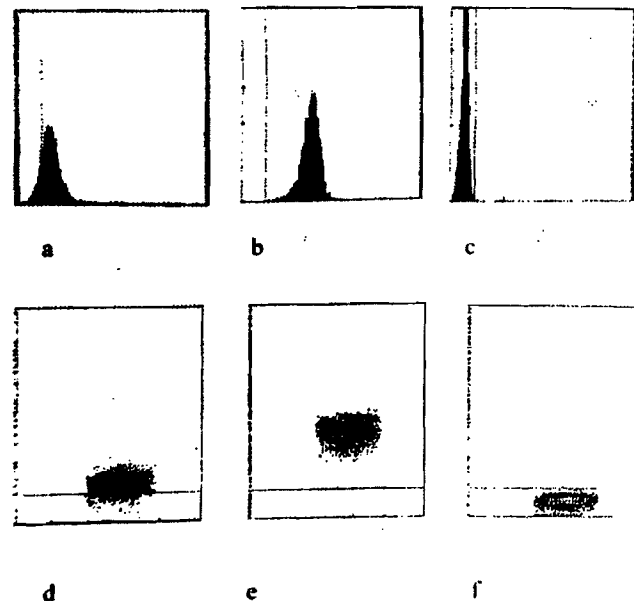


Fig. 5. Flow cytometry analysis showing binding to HPB MLT cells (a–c) or peripheral blood lymphocytes (PBL; d–f) of chimeric recombinant anti-CD6 antibodies present in the milk of transgenic rabbit F012 (a,d), murine hybridomaderived anti-CD6 antibodies (b,e) and a non-related human antibody as negative control (c,f).

### 8.2. $\beta$ -casein promoter

A fragment of the rabbit  $\beta$ -casein promoter was used to target the production of human interleukin-2 (hIL-2) to the milk of transgenic rabbits in a pioneer experiment by Bühler et al. [56]. Four transgenic rabbit lines were tested. Their milk contained biologically-active hIL-2 at a concentration of 50–340 mg/l milk.

### 8.3. Glimpsing the future

As noted above a variety of important human therapeutic proteins have been secreted in transgenic rabbit milk, and in many cases biological activity have been confirmed and the proteins have been purified almost to the homogeneous state. However, at present there are still limitations for the routine production of recombinant proteins in the milk of transgenic rabbits.

The mammary gland can perform a range of post-translational modifications that are often essential for biological activity. These include disulfide bridge formation, gamma carboxylation of glutamic acid residues; N- and O- linked glycosylation, amidation, subunit assembly and multimerization of complex proteins. However, not always the desired modification has been achieved, as it was the case for glycosylation of the hEPO [46]. In other transgenic mammals similar problems have been faced such as defective O-linked glycosylation of human bile-salt stimulated lipase [57], incomplete proteolytic processing of human protein C in swine mammary gland [58] and absence of phosphorylation of human  $\alpha$ -lactalbumin in transgenic cattle [48]. These facts indicate that there is still research to be carried out in order to control and monitor the posttranslational mechanisms governing these events in the mammary gland of transgenic animals.

The expression levels and the tissue specificity of the promoters used are still too little predictable. One approach to achieve strict spatio-temporal pattern of expression from genes of interest is the use of large gene constructs providing extensive sequences flanking the coding unit of the gene in order to avoid unwanted side effects of transgene expression. Replacement of murine  $\alpha$ -lactalbumin gene by its human counterpart was achieved in knock-out mice. The homozygous mice expressed high levels of the human gene protein in their milk [59]. A very interesting approach therefore, will be the insertion of a gene of a pharmaceutical protein by homologous recombination leaving intact all the flanking regulatory sequences. Ideally, high levels of strict tissue-specific expression could be attained. Recently, transgenic rats were generated from a yeast artificial chromosome (YAC) vector containing 210-kb of the human  $\alpha$ -lactalbumin gene and regulatory sequences [60]. In this experiment, high-level, position independent expression was found in all the transgenic lines

tested. The feasibility of YAC transgenesis in rabbits has also been demonstrated [61]. Therefore, the cloning in YACs of megafragments of milk protein genes, with its subsequent microinjection into one-cell embryos could be used as a powerful tool for improving the efficiency of generation of high-expressing transgenic rabbits.

Another possibility is the addition of heterologous regions, e.g. locus control regions (LCRs) or matrix attachment regions (MARs) [62,63], known to be responsible for maintaining a stable tissue-specific open chromatin structure. It was shown in transgenic mice that flanking sequences from the chicken lysozyme A element, containing MAR were able to relieve the severe position effects imposed to a 1.0-kb mouse WAP promoter [63]. Without doubt, an exciting development will be the in vitro establishment of totipotent cells from rabbits and their subsequent use in cloning and transgenesis experiments. The availability of this technique will give new impetus to gene transfer, because it will not only provide the possibility of additive gene transfer and homologous recombination but will also notably reduce problems such as low efficiency, non-expression of transgenes or insertional mutations.

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# The Mammary Gland as a Bioreactor: Expression, Processing, and Production of Recombinant Proteins

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A variety of transgenic animal species are being used to produce recombinant proteins. The general approach is to target the expression of the desired protein to the mammary gland using regulatory elements derived from a milk protein gene and then collect and purify the product from milk. Promoter sequences from a number of different milk protein genes have been used to target expression to the mammary gland, although significant problems remain with regard to achieving transgene expression levels consistent with commercial exploitation. The mammary gland appears to be capable of carrying out the complex posttranslational modifications, such as glycosylation and  $\gamma$ -carboxylation required for the biological activity and stability of specific proteins. Effective purification protocols have been established and products produced by this route have now entered clinical trials.

**KEY WORDS:** Transgenic; livestock; mammary gland; protein production.

## INTRODUCTION

Human proteins have been used in medicine for many years. Most notable is the wide variety of plasma products supplied by the blood fractionation industry. These include immunoglobulin preparations, serum albumin, and concentrates of the clotting factors VIII and IX. In the past human proteins from sources other than blood have been extremely limited by supply. For example, until relatively recently, the only source of human growth hormone was pituitary glands procured from human cadavers.

Recombinant DNA technology has revolutionized the means of production of therapeutic proteins and no longer are supplies of a particular product limited by the supply of human material. Genes encoding a large number of human proteins have now been cloned, including insulin, growth hormone, protein C

(hPC),<sup>3</sup> tissue plasminogen activator (htPA) and factors VII, VIII (hfVIII), and IX (hfIX). In some cases the expression of protein from these cloned genes has been accomplished in microorganisms, for example, in the production of human growth hormone and human insulin. For many proteins, however, microorganisms such as yeast and bacteria are not suitable because they require posttranslational modifications of their structure for biological activity or stability. Such modifications include the covalent addition of sugar residues, the chemical modification of certain amino acids and the specific cleavage of protein precursors. Bacteria and yeast are unable to carry out these modifications appropriately, but they are performed by mammalian cells. Consequently, there has been an intense effort to understand and optimize protein production in mammalian cell culture systems.

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<sup>3</sup> Abbreviations: beta-lactoglobulin (BLG); human  $\alpha_1$ -antitrypsin (h $\alpha_1$ AT); locus control region (LCR); whey acidic protein (WAP); human factor VIII (hfVIII); human factor IX (hfIX); human protein C (hPC); human tissue plasminogen activator (htPA); chloramphenicol acetyl transferase (CAT); untranslated region (UTR).

The culture of mammalian cells on a commercial scale is expensive and technically demanding. In 1982 an alternative to the production of proteins in cell culture was suggested by Palmiter and his colleagues (1). These workers described the production of transgenic mice carrying copies of the rat growth hormone gene. In these experiments the cloned rat gene had been introduced into the germline by the direct injection of DNA into the pronuclei of the fertilized mouse eggs. A proportion of the animals that developed from these eggs carried copies of the foreign DNA integrated at a site on one of the chromosomes. Prior to injection the rat sequences had been fused to regulatory DNA sequences from another gene, the metallothionein gene. This gene is expressed predominantly in the liver and, indeed, the metallothionein promoter sequences determined the site of expression. Large amounts of growth hormone were synthesized in the liver and the protein was secreted into the plasma, causing the animals to grow more rapidly and to a greater final size.

The plasma levels of the foreign protein were up to 100-fold higher than had been reported for growth hormone produced by genetically engineered bacteria or mammalian cells at that time and so the notion of producing therapeutic proteins in transgenic animals was born.

Work in transgenic mice, such as that described earlier for growth hormone, demonstrated the feasibility of targeting a particular protein to a specific tissue using the appropriate regulatory elements. We argued more than a decade ago that the mammary gland was the obvious tissue to target the expression of foreign proteins (2). Milk protein genes are expressed specifically and at high levels, and milk is collected easily and without detriment to the animal. In this paper I shall review the progress that has been made during the last decade in the development of this approach primarily for the production of human proteins, highlighting both the challenges and the successes.

## PRODUCING TRANSGENIC ANIMALS

**Pronuclear Injection.** The basic techniques for producing transgenic animals were established in the mouse at the beginning of the 1980s (3). They involve the direct pronuclear injection of DNA sequences into the fertilized egg, followed by surgical implantation into the reproductive tract of a hormonally-primed recipient foster mother.

Transgenic mice have been used widely to assess the feasibility of many of the aspects of protein production in the mammary gland (see later). Indeed, the first paper describing the production of a recombinant human protein in milk, tissue plasminogen activator (htPA) was in transgenic mice (4). Mice, however, produce only small quantities of milk and, therefore, the realization of this approach has necessitated the development of techniques for generating large transgenic mammals. The pronuclear injection methodology has been adopted from the mouse, although some modifications have been required to take account of species differences. Successful gene transfer by this route has been described for all the major commercial livestock species including sheep (5), goats (6), pigs (7), and cows (8), as well as rabbits (9).

Working with larger domestic animals poses a number of technical and logistical problems. The recovery of suitable numbers of pronuclear eggs and their transfer following microinjection requires a large number of donor and recipient animals. The zygotes for injection may be obtained by superovulation of donor females or they may be produced by the *in vitro* maturation and fertilization of oocytes obtained from the ovaries of slaughtered beasts. This eliminates the need for maintaining large numbers of donor animals and this approach is routinely employed in the generation of transgenic cattle (8). Microinjection of eggs from livestock is complicated by their relative opacity. In sheep and goats, careful microscopy using Nomarski differential interference optics is required to visualize the pronuclei. Bovine and porcine zygotes must be centrifuged to sediment the cytoplasmic lipid vesicles to enable visualization of the pronuclei although this does not appear to reduce embryo viability significantly (Fig. 1).

The efficiency of gene transfer, in terms of the number of eggs injected and transferred vs. the number of transgenic animals produced is usually between 1% and 2% (10). Once integrated in the germline the transgenes are usually, but not always, transmitted to the next generation. Rather disappointingly, there has been little improvement in the efficiency of this procedure during the last 10 years and this figure is similar to those given in the first reports describing the generation of transgenic livestock (5, 7). A number of workers have attempted to improve the overall efficiency of the injection procedure, for example, by devising cytoplasmic injection strategies, but with little success (11). Other groups have attempted to devise strategies to preselect embryos carrying the integrated transgene.

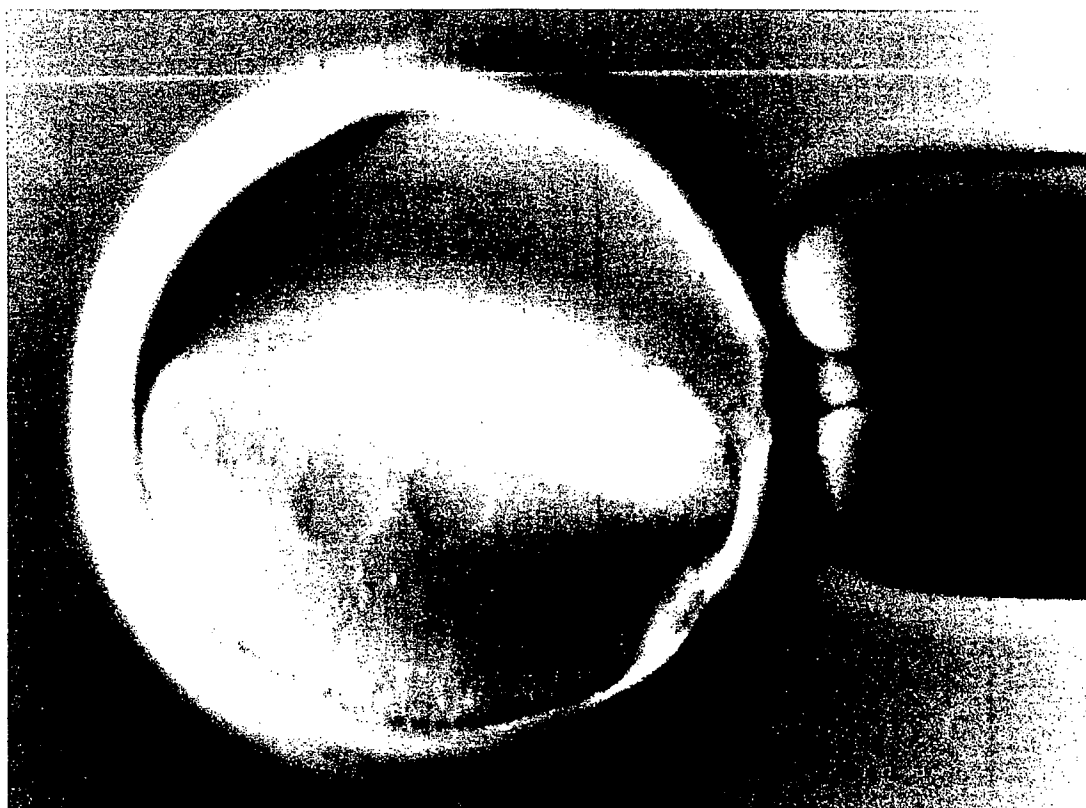


Fig. 1. Visualization of pronuclei. In livestock, visualization of the pronuclei is difficult because of the presence of dense lipid granules in the cytoplasm of the zygote. This problem can be resolved by centrifuging (12,000g/5 min) the zygotes prior to injection after which both male and female pronuclei become clearly visible. The holding pipette is on the right and the two pronuclei are clearly evident toward the top of the sedimented cytoplasm.

Transferring only positive embryos would dramatically reduce the number of recipient animals required in an experiment. However, working with the small amount of material available from embryo biopsies, it has proved impossible to reliably distinguish integrated from nonintegrated transgenes.

**Cell-Based Transgenesis.** A major bottleneck in the production of transgenic livestock has been the low efficiency of generating transgenic founders. The problem stems from the difficulty of having to work with the zygote as the cell into which the DNA is introduced. Zygotes can't be multiplied (as can conventional cells in culture) and they may only be cultured *in vitro* during the earliest stages of embryonic development. A radical improvement would be to accomplish the required genetic manipulation in conventionally cultured cells which could be used at a later stage to generate animals. Embryonic stem (ES) cells in mice are now widely used to manipulate the mouse genome. These are cells derived from the early

embryo. They can be propagated in culture and, in the presence of the necessary growth factors, retain their totipotency. When injected back into a host blastocyst their descendants contribute to the tissues of the ensuing chimera including, most importantly, the germline. They are very tractable to DNA manipulation and have been used widely to target specific changes, such as gene deletions or replacements, in the genome. A selectable marker gene such as that encoding neomycin phosphotransferase (*neo*) is simultaneously introduced to allow selection of positive cells. In principle, then, ES cells would seem the ideal candidate from which to develop an alternative route to transgenesis in livestock. Despite intensive efforts, however, no validated ES cells (i.e., cells that will contribute to the germline) have been described for any species of livestock (12). Indeed, ES cells have not been described for any species other than the mouse, and in this species their generation appears to be limited to just a handful of inbred lines.

Until recently attempts to develop a cell-based system for transgenesis in livestock foundered on the failure to develop ES lines. Exciting, and largely unanticipated, developments in animal embryology, pioneered by Ian Wilmut and Keith Campbell at the Roslin Institute are, however, set to revolutionize transgenic technology in livestock. The key finding from these workers was that viable animals could be generated when nuclei from differentiated sheep cells that had been maintained in *in vitro* culture were transferred into enucleated oocytes (13). Contrary to dogma, the nuclei from these cells were not irreversibly determined but could be re-programmed to support full development to term and beyond. The breakthrough may be due to the use of quiescent cells in the G0 phase of the cell cycle as the nuclear donors, as it is thought that these nuclei may be more readily programmable. The first experiments were carried out using cultured cells derived from the early embryo (13). Subsequent experiments showed that other cell types including foetal fibroblasts and, most dramatically of all, adult mammary epithelial cells could be successfully used as the source of nuclei (14).

The ability to clone farm animals from cultured cells by nuclear transfer has profound implications for the generation and propagation of transgenic livestock. Recently, scientists at Roslin and PPL Therapeutics have described the production of transgenic sheep by nuclear transfer from transfected fetal fibroblasts (15). In these experiments the fetal fibroblasts derived from polled dorset sheep were cotransfected with a *neo* selectable marker gene and a gene encoding hflX linked to regulatory elements from the sheep BLG gene. Two cloned transfectant cell lines as well as a population of *neo* resistant cells were used as donors in nuclear transfer experiments. Six transgenic lambs were liveborn; the three produced from the cloned cells contained both hflX and *neo* transgenes whereas those from the uncloned population contained the marker gene only.

These experiments demonstrate for the first time the feasibility of using a cell-based route for transgenesis in large animals. Even in these early experiments this approach appears to be more efficient than pronuclear injection; it was estimated that less than half the animals that would have been required by conventional pronuclear injection were used (15). Nuclear transfer enables the cloning of identical copies of transgenic animals and so it may be possible to rapidly expand producer populations by using this approach rather than conventional breeding (Fig. 2). If gene targeting

## Conventional Transgenesis

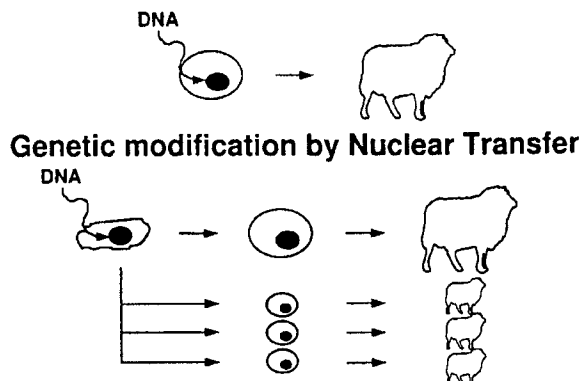


Fig. 2. Comparison of conventional transgenesis and cell based transgenesis using nuclear transfer. Using conventional transgenesis DNA is introduced into the 1-cell embryo. Transgenic status is usually determined after birth. In cell-based transgenesis the DNA is introduced into cultured cells using a selectable marker and positive cells selected prior to nuclear transfer into an enucleated oocyte (15). If cloned cells are used, then the transgenic status of the animals that are generated is guaranteed. Further nuclear transfer from the cells could generate a clonal population of transgenic animals. If gene targeting strategies can be adapted to this procedure, then the possibility of carrying out precise genetic manipulations in livestock is opened up.

by homologous recombination can be achieved in donor cells such as fibroblasts, then the way is opened up for making much more precise manipulations to the genome. Pronuclear injection gives the experimenter no control over the site or structure of the transgene integration site with major effects on the efficiency and reliability of expression (see later). Gene targeting would enable transgenes to be inserted as single copies at defined sites in the genome to avoid positional effects due to the site of integration and the tandem repeat nature of conventionally generated arrays (16). In some circumstances it may be appropriate to use endogenous promoters by targeting the coding sequences downstream, rather than introducing the conventional hybrid construct. Although nuclear transfer technology has been only proven and properly reported in sheep, there are already press reports of cattle cloned by nuclear transfer from cultured cells and it can only be a matter of time before this technology becomes more widely adopted.

**Choice of Species.** Notwithstanding the route for transgenesis that can be used there are clear decisions that must be made regarding the choice of species for protein production. These relate to the quantity of protein product that will be required and the timescale

for production. Two key considerations are the volume of milk produced per lactation and the generation time. Thus rabbits produce approximately a liter of milk per lactation and can be bred many times during a single year and a doe can produce up to 10 liters of milk during this period. By contrast a cow can produce more than 10,000 liters per lactation. However, to generate a naturally lactating transgenic cow requires a minimum time period of 2.5 years (9 months gestation; 1 year to sexual maturity; 9 months gestation) and possibly up to 2 years longer if the original founder animal was a bull. Sheep and goats lie in between these extremes. They both can produce several hundred liters of milk per lactation and the time to the first lactation is approximately 1.5 years. It is not surprising, therefore, that it is in these two species that commercial levels of protein production were the first to be described (Table I) and it is these two species that have formed the first producer populations for the relevant commercial enterprises now exploiting this technology. Pigs have also been proposed as possible producer animals; they can produce in excess of 100 liters of milk per lactation and the time to the first lactation is about 15 months. They are one of the more tractable large animal species in terms of conventional transgenesis but are not conventional dairy animals, and obtaining milk from them is difficult and unpredictable. Nevertheless, a number of complex proteins including protein including human protein C and human factor VIII have been expressed in swine milk (Table I).

## TARGETING GENE EXPRESSION TO THE MAMMARY GLAND

**Milk Protein Genes.** Milk comprises a relatively small number of major proteins that are secreted specifically by the mammary gland. These proteins are grouped into two major classes, caseins and whey proteins. In cattle, for example, there are four caseins ( $\alpha$ S1,  $\alpha$ S2,  $\beta$ , and  $\kappa$ ) and two whey proteins (BLG and  $\alpha$ -lactalbumin). The genes encoding these proteins are single copy and are transcribed at high levels specifically in the mammary gland during pregnancy and lactation. A large number of milk protein genes from a variety of different species have been cloned and characterized (17). Some of these genes have been introduced into transgenic mice and their expression assessed in the mammary gland. In some cases very high levels of expression have been achieved. For example, for goat  $\beta$ -casein (18) and sheep BLG (19) levels in excess of 20 mg/ml have been reported (see also Ref. 20).

**Promoters.** The specificity and high levels of expression of endogenous milk protein genes in the mammary gland (as well as selected milk protein transgenes) indicated that regulatory elements from these genes could be used to direct the expression of foreign genes to this tissue. During the last 10 years a number of different milk protein gene promoters have been used to target expression of a variety of human therapeutic proteins to the mammary gland

Table I. Expression of Recombinant Human Proteins in the Milk of Transgenic Livestock

| Species | Protein                    | Promoter                               | Expression  | Ref.  |
|---------|----------------------------|--|-------------|-------|
| Rabbit  | hIL 2                      | rabbit $\beta$ -casein                 | 0.45 ug/ml  | 9     |
|         | hIGF-1                     | bovine $\alpha$ S <sub>1</sub> -casein | 1.0 mg/ml   | 59    |
|         | h $\alpha$ <sub>1</sub> AT | goat $\beta$ -casein                   | 4.0 mg/ml   | 54    |
|         | hPC                        | ovine-BLG                              | 0.7g mg/ml  | 54    |
| Sheep   | hfIX                       | ovine BLG                              | 0.025 ng.ml | 60    |
|         | hPC                        | ovine BLG                              | 0.3 mg/ml   | 53,55 |
|         | hfibrinogen                | ovine BLG                              | 5.0 mg/ml   | 53,55 |
|         | h $\alpha$ <sub>1</sub> AT | ovine BLG                              | 35.0 mg/ml  | 25    |
| Goat    | htPA                       | goat $\beta$ -casein                   | 6.0 mg/ml   | 6     |
|         | hATIII                     | goat $\beta$ -casen                    | 14 mg/ml    | 54    |
|         | h $\alpha$ <sub>1</sub> AT | goat $\beta$ -casein                   | 20 mg/ml    | 54    |
|         | Anti-cancer Mab            | goat $\beta$ -casein                   | 10 mg/ml    | 54    |
| Pig     | hPC                        | mouse WAP                              | 1 mg/ml     | 50    |
|         | hPC                        | ovine BLG                              | 0.75 mg/ml  | 55    |
|         | hfVIII                     | mouse-WAP                              | 3 mg/ml     | 51    |
| Cattle  | ha-lac                     | human a-lact.                          | 2.4 mg/ml   | 55    |

<sup>a</sup> The maximum levels reported for each protein are tabulated against species and the promoter elements used to drive expression. Adapted from Ref. 53.

(20). Many of these experiments have been carried out using transgenic mice, which are often used as a model to test construct design prior to transfer to larger species. Overall, the results are equivocal; most of the promoters tested did target expression to the mammary gland to some degree but with wide variations between different experiments. Most appeared to exhibit specific expression in the mammary gland although examples of ectopic transgene expression have been reported (21). In some cases very high levels of expression of the target protein were reported. For example, Meade and co-workers (22) reported a 1–2 mg/ml concentration of urokinase using the bovine  $\alpha$ S1 promoter and Archibald and co-workers (21) 7–8 mg/ml of human  $\alpha$ 1-antitrypsin (h $\alpha$ AT) using the ovine BLG promoter.

**Construct Design.** The large number of experiments carried out in transgenic mice and livestock clearly demonstrated the feasibility of this approach for the production of foreign proteins in the mammary gland. Nevertheless, there is still tremendous variability in the efficiency of transgene expression even when the same regulatory sequences are used.

In a large study we compared the expression levels of a number of different transgenes driven by the BLG promoter that either contained or lacked introns (23). The results were quite clear in that the transgenes containing most or all of their natural introns were expressed much more efficiently than their intronless counterparts; this was consistent with observations from other laboratories (24). For example, constructs comprising the BLG sequences fused to the cDNA sequences encoding h $\alpha$ 1AT were poorly, if at all, expressed. By contrast, a construct comprising the same BLG promoter elements used in the cDNA constructs but fused to a genomic minigene encoding human  $\alpha$ 1AT was expressed very efficiently and mice expressing 7–8 mg/ml of the human protein were produced (21). This same construct was introduced into sheep; one founder animal exhibited expression at incredibly high levels—more than 30 mg/ml (25), clearly showing the feasibility of the approach for producing large amounts of a given human protein.

The identification of regulatory regions outside the conventional promoter region that enhance transgene expression, first exemplified in the  $\beta$ -globin gene cluster (26) and now termed locus control regions (LCRs) raises the question as to whether similar dominant regulatory elements are present at milk protein gene loci which could be incorporated into constructs to augment expression. At the time of writing, how-

ever, no milk protein LCRs have been described. In some cases, however, elements from other genes have been evaluated. The A element from the chicken lysozyme gene has been incorporated into a WAP transgene (27). In transgenic mice the WAP gene is expressed in only about 50% of the lines generated. Furthermore, its regulation during pregnancy and lactation is different from that of the endogenous gene and is quite variable between lines. By contrast, all 11 lines in which the WAP transgene was juxtaposed to the A elements expressed the transgene and most showed appropriate developmental regulation. However the same elements failed to improve expression when incorporated into a BLG-CAT construct (16).

**Position Effects.** Most transgenes are strongly influenced by their site of integration in the host chromosome. We have argued that the vicinity of an actively expressed milk protein gene might provide a site of integration permissive for the expression of a normally inefficiently expressed transgene. In mice this could be accomplished by using ES cells and this general approach has been exemplified recently by Bronson and co-workers (28), who showed that targeting a transgene into a defined site at the hypoxanthine phosphoribosyl transferase (HPRT) locus significantly improved the reliability of its expression. Gene targeting has yet to be demonstrated in livestock and, therefore, we chose an alternative approach that involves the co-integration of two transgenes at a single chromosomal site. In this approach poorly expressed BLG derived transgenes designed to express h $\alpha$ 1AT or hFIX were co-integrated with the efficiently expressing unmodified BLG transgene (29). This strategy resulted in significant improvement of the efficiency and frequency of expression of these constructs in the mammary gland. The “rescue” effect appears to require the functionality of the co-integrated BLG gene and BLG genes from which the promoter has been removed do not confer this effect (30).

**Transgene Silencing.** Recently, a number of laboratories have reported that transgenes are susceptible to potent silencing effects that manifest as a heterocellular or mosaic pattern of expression reminiscent of classical Position Effect Variegation observed originally in *Drosophila* (31). We have observed similar effects in the mammary gland and lines expressing the BLG transgene can exhibit highly variegated patterns of expression (32). The mechanism behind these silencing effects is not fully understood, but a critical feature appears to be the multicopy repeat nature of

transgenes and the existence of repeat sensitive transcriptional repression has been postulated (33).

Silencing may also be dependent on the nature of the transgene sequences themselves. Thus it has been known for many years that cDNA and prokaryotic reporter sequences are difficult to express in transgenic animals (see earlier). By co-injecting such cDNA constructs with the efficiently expressed BLG gene the frequency and level was improved. In an extension to this approach we linked the two genes together prior to injection to test whether we could improve the reliability of the approach (34). Enhanced reliability was not achieved, and to our surprise the main effect observed was the complete silencing of the BLG gene in the majority of the lines generated (Fig. 3). These experiments were done with both mammalian cDNA (hFIX) and a prokaryotic reporter (CAT) and both these sequences efficiently silenced the adjacent BLG gene, suggesting that such sequences can serve as active foci gene for repression in the genome.

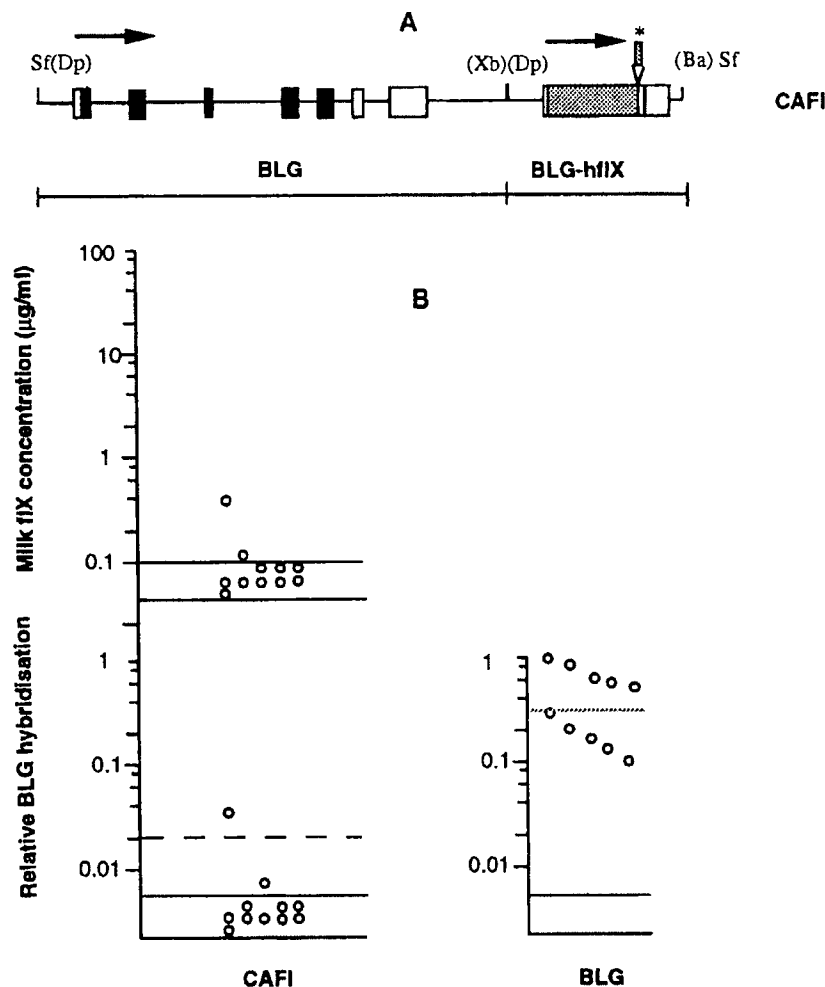
## RNA PROCESSING

Most efforts with regard to improving transgene expression in the mammary gland have focused on trying to improve transcription rates and, relatively speaking, little effort has gone into issues surrounding RNA processing. For the most part this is understandable as many of the basic mechanisms of mRNA metabolism such as capping, polyadenylation and export from the nucleus are carried out by all cells. Nevertheless, there are some processes that do vary between different cell types and to achieve efficient expression it may be necessary to optimize these in the long run.

*Splicing.* Consensus splice sites (35) are distributed throughout pre-mRNA sequences, yet only a specific subset of these are activated during splicing in the nucleus. The selection of splice sites in pre-RNA is poorly understood but is known to be determined by both *cis* and *trans*-acting factors. When hybrid constructs are transcribed quite novel pre-mRNAs are often generated, particularly if cDNA or minigenes are employed and this could lead to the activation of cryptic sites. Unfortunately, the rules for predicting the likelihood of a particular donor or acceptor site becoming activated are virtually nonexistent; in many ways it becomes a matter of trial and error, although experiments in appropriate cell lines may provide useful screening for aberrant splicing events (36).

Activation of cryptic splice sites can have disastrous effects. In our attempts to express hFIX in the milk of transgenic mice we achieved relatively high steady state mRNA levels of the hFIX cDNA transgene by co-integrating it with the unmodified BLG gene (29). Virtually no hFIX protein was detected in the milk of these animals, however. On closer inspection of the mRNA generated in these experiments it was found to be ~ 450 nt shorter than predicted. Cloning by RT-PCR and sequencing of the transgene mRNA revealed a 462 bp deletion within the hFIX cDNA sequences encompassing the C terminal amino acids and 3' UTR (37). The deletion was flanked by consensus donor and acceptor splice sites, strongly suggesting the activation of cryptic splice sites (Fig. 4). A transgene in which the 3' acceptor site was removed was constructed and tested in transgenic mice. Pre-mRNA transcribed from this construct was no longer spliced aberrantly and relatively high levels of hFIX were secreted into the milk (37). Since hFIX cDNA appears to be correctly spliced when it is expressed in the liver (38) it seems likely that the aberrant splicing observed in the mammary gland reflects differences in *trans*-acting splicing factors between these two tissues.

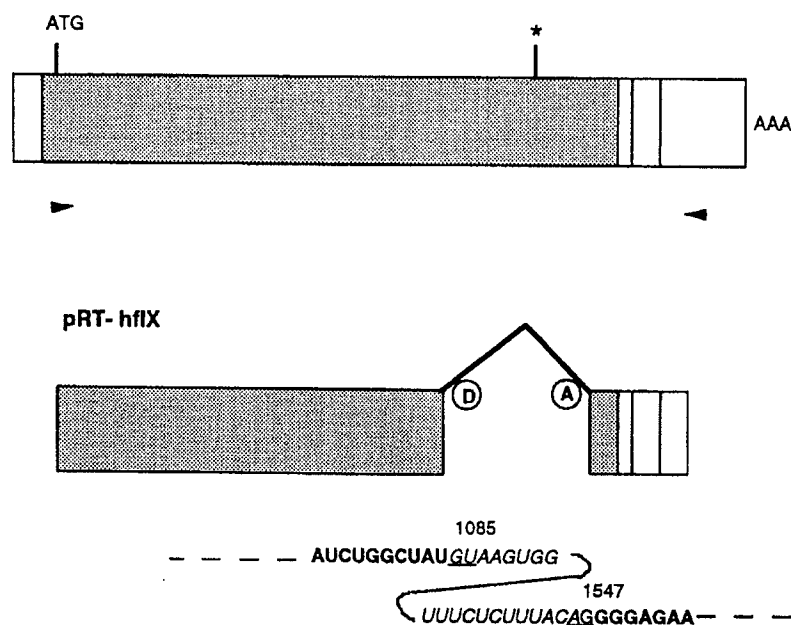
*mRNA Stability.* Although mRNA stability is a key feature in determining expression levels, the mechanisms that determine decay rates are only just beginning to be understood. At least three mechanisms mediate mRNA stability, poly A shortening, translational impairment and endonucleolytic cleavage (reviewed in Ref. 39). A large number of *trans*-acting factors that play a role in mRNA stability have been described whose functions vary from nucleolytic activity to more complex roles such as targeting mRNAs to specific degradative pathways. Some of these factors act globally while other are restricted to specific mRNA subsets. Interestingly, early work on the expression of casein genes suggested an important role for hormonally-mediated stabilisation of mRNA as a means of regulating expression levels in the mammary gland (40). *In vivo* in the lactating gland milk protein mRNAs exhibit very high steady state levels (5–10% total poly(A) mRNA in some cases) and it is tempting to speculate that mechanisms which prevent decay contribute to these levels. Little effort, however, has gone into this area with respect to improving transgene expression, even though a number of nucleotide elements have been described which apparently increase the cytoplasmic accumulation of eukaryotic mRNAs (41).



**Fig. 3.** Transgene silencing. (A) A two-transgene construct termed CAFI was built which comprises the BLG gene linked to a BLG-hfIX transgene and was introduced into transgenic mice. Open and full-shaded boxes, BLG exons; hatched box, hfIX cDNA sequences; line, BLG 5' and 3' flanking and intronic sequences. (B) Transgene expression profiles. The frequency and level of expression of various transgenes in the mammary gland are shown. Each circle represents an independently generated mouse line and usually represents the average level of expression determined in the G1 generation. Circles below the ordinate represent lines in which no transgene expression could be detected. In the transgenic lines carrying the CAFI construct (left) two/eleven lines showed low levels of fIX expression. Only two CAFI lines exhibited detectable BLG expression. By contrast when the same BLG gene used in the CAFI construct was introduced as a single transgene (right) all ten transgenic lines exhibited high levels of expression. Adapted from Ref. 34.

**Translational Control.** mRNA translatability and stability are intimately linked and mRNAs in which translation is impaired often have reduced stability (39). For example, in addition to being a modulator of mRNA turnover the poly (A) status of an mRNA species also determines its translational efficiency. Most of our understanding of translational control

comes from work in model systems such as *Xenopus* oocytes, yeast and cultured cells and little of this information has been applied to improving gene expression in transgenic animals. mRNA translation is affected by the structure encompassing the AUG translational initiation codon (42) and, in some instances, it may be appropriate to modify these sequences to more closely



**Fig. 4.** Aberrant transgene mRNA splicing. hIFX cDNA sequences were targeted to the mammary gland using the BLG promoter. The predicted hIFX mRNA structure is shown. Northern blotting experiments indicated that the transgene mRNA was ~450 nt shorter than expected (29). These transcripts were amplified by RT-PCR at the primer sites indicated. Sequencing the amplified product showed a 462 bp deletion at the 3' end of the hIFX sequences. Inspection of the breakpoints showed them to be flanked by consensus donor and acceptor splice sites indicating the activation of these cryptic splice sites in the mammary gland. Adapted from Ref. (37).

match the so-called "Kozak consensus." Other sequence elements which modulate translation have been identified within the coding region or 3' untranslated regions (43) and, in principle, it may be possible to incorporate such elements into the transgene constructs to improve expression levels

One of the principal reasons for using the mammary gland of transgenic animals for protein production is that many therapeutic proteins require post-translational modifications for biological activity and/or stability which nonmammalian cells do not carry out correctly. Human proteins expressed in the mammary gland are being synthesized in a heterologous system, both in terms of species and cell type with unpredictable consequences for the nature and the degree of

posttranslational modifications. Therefore, once acceptable expression levels of a protein have been achieved in milk, a critical next step is to characterize the posttranslational modifications as well as the overall biological activity of the protein.

**Glycosylation.** Glycosylation is a crucial post-translational modification which can affect the solubility, stability, biological activity, and immunogenicity of many proteins. For example, glycoproteins containing high mannose or desialylated structures may have a short half-life in the circulation due to high mannose and asialoglycoprotein receptors in the liver.

Human  $\gamma$ -interferon contains two N-linked glycosylation sites at Asn 25 and Asn97. James *et al.* (44) demonstrated that  $\gamma$ -interferon expressed in the mammary gland of transgenic mice contained complex sialylated and fucosylated glycans at Asn 25 but high mannose structures at Asn 97 which could make such a protein susceptible to clearance by mannose receptors.

Denman and co-workers studied the glycosylation of a variant form of human tissue plasminogen activator expressed in the mammary gland of transgenic goats (45). They found lower levels of galactose, N-acetyl glucosamine and sialic acid in the transgenic protein as compared to the native protein. They also detected N-acetyl galactosamine residues which are not present in the native protein and could potentially lead to problems of immunogenicity. The bovine mammary gland is also known to process some N-linked glycoproteins aberrantly including the incorporation of terminal N-acetyl galactosamine residues and more complex branched mannose structures (46).

We have studied the glycosylation status of human  $\alpha_1$ AT produced in the mammary gland of transgenic mice (21).  $\alpha_1$ AT has three N-linked glycosylation sites and the glycosylation status of these is an important consideration for its pharmacokinetics. The material from transgenic mice exhibited biantennary and triantennary structures but exhibited a higher level of fucosylation than the human plasma derived material, although high mannose and hybrid structures were not detected (47). Given the variable and rather unpredictable nature of glycosylation, as illustrated by these examples, it seems likely that it will be absolutely essential to characterize in detail the glycan structures of each potential product prior to further development.

**$\gamma$ -Carboxylation.** A number of human plasma proteins require the  $\gamma$ -carboxylation of certain glutamic acid residues for biological activity. The  $\gamma$ -carboxylated residues serve to bind calcium which is an essential cofactor for their activity. In the case of hFIX a cluster of 12 Glu residues near the N terminus must be  $\gamma$ -carboxylated for full biological activity (48). In our studies on the expression of hFIX in the mammary gland of transgenic mice we have used a monoclonal antibody specific for this calcium-binding Gla domain to purify the hFIX produced in the milk (37). This technique resulted in recovery of about half the hFIX from the starting material, all of which was shown to be fully active in a clotting assay. The mammary gland thus appears to be capable of carrying out the  $\gamma$ -carboxylation of hFIX quite efficiently. This finding is particularly gratifying, since  $\gamma$ -carboxylation has not been reported for any of the endogenous proteins made in the gland. Another  $\gamma$ -carboxylated protein, human protein C (hPC), has also been made in the milk of transgenic animals. In transgenic mice propeptide cleavage (see later) and  $\gamma$ -carboxylation were incomplete and only trace activity was detected (49). In transgenic pigs hPC was present in milk at levels

between 0.5 and 1.0 mg/ml but only 30–60% of this material was fully active (50).

**Other Modifications.** Recently, the production of one of the largest and most complex plasma proteins, factor VIII, has been reported in the milk of transgenic swine (51). This protein requires N- and O-linked glycosylation and tyrosine sulfation for function. Although only quite low levels of the protein were obtained (3  $\mu$ g/ml) it appeared to be correctly modified and exhibited biological activity.

**Proteolytic Maturation.** A number of potential therapeutic proteins including albumin and vonWillebrand's factor are first synthesized as inactive preproteins which must be cleaved by specific endoproteases present in the endoplasmic reticulum and Golgi to yield the mature form of the protein. Functional human protein C (hPC) is converted from a single chain to a two chain molecule. When this protein was expressed in the milk of transgenic mice (49) or pigs (50) it was only partially processed and a mixture of the pro-single chain and the mature heavy and light chain was observed. In an elegant experiment, Drew *et al.* (52) devised an approach to enhance proteolytic maturation in the mammary gland. The serine protease furin has been shown to process a variety of proproteins in cultured cells. Double transgenic mice in which the expression of both hPC and furin were targeted to the mammary gland by the WAP promoter were generated. Co-expression of furin with hPC in the mammary gland resulted in efficient conversion of the pro-hPC precursor to the mature protein with cleavage occurring at the correct site. It should be noted that there may be significant species differences with regard to proteolytic processing capacity in the mammary gland. Thus although both mice and pigs do not appear to process hPC fully (in the absence of furin expression), transgenically derived material from sheep, expressed at levels up to 1 g/liter appears to be fully processed and is as active as plasma derived hPC in *in vitro* coagulation assays (53).

**Multimeric Proteins.** A number of potential protein products comprise two or more polypeptide chains encoded by separate genes. In these cases both genes must be expressed in the same cells in the mammary gland and the polypeptide chains processed, folded and appropriately assembled in the endoplasmic reticulum and Golgi apparatus prior to secretion. Monoclonal antibodies (Mabs) consist of separately encoded heavy and light chains and high levels of Mab expression (up to 5 mg/ml) have been reported in the milk of transgenic goats (54). Perhaps the most impressive

example in this regard is the high level of recombinant human fibrinogen which has been reported in sheep milk (53, 55). Fibrinogen comprises six polypeptide chains—dimeric  $\alpha$ ,  $\beta$  and  $\gamma$  chains. The three genes encoding the different chains were targeted to the mammary gland of transgenic sheep using BLG regulatory elements. Concentrations up to 5 g/liter were obtained in the milk, more than 1000-fold greater than was ever achieved in cell culture and the transgenic derived material appeared to be fully functional in a clotting assay.

## DOWNSTREAM PROCESSING

A number of transgenic livestock expressing substantial amounts of human therapeutic proteins have now been produced (Table I). In many ways this is only just the beginning and many technical and regulatory hurdles must be crossed before a product that is suitable for administration to patients is realized.

*Scale up.* One of the advantages of pharmaceutical protein production in animals is that it offers a direct route for the scaling up of production by breeding producer populations. If the founder animal is female then the initial expression analysis can be carried out in this generation. Subsequent breeding from females has some limitations in terms of the progeny obtainable although using techniques such as transvaginal echoscopy to recover large numbers of immature oocytes *in vivo* (56), *in vitro* maturation and fertilization and embryo transfer can generate relatively large numbers of progeny. If the founder animal is male then daughters must be bred and expression analysis is delayed until their lactation in the next generation, although an early assessment of expression can be achieved by inducing lactation in prepubertal animals.

Breeding studies are important to determine the inheritance as well as the genetic and expression stability of the transgene locus. Transgenic sheep carrying a transgene targeting the expression of  $\alpha_1$ AT to milk were bred (25, 26); of the six founders only four transmitted the transgene to the next generation. Of these four one female produced progeny with variable copy numbers of the transgene; this appeared to be the result of genetic rearrangement rather than the segregation of independently generated transgene integration sites (57). The three other founders all transmitted their transgene locus in as stable a fashion. From one of these (a male) a number of transgenic daughters were obtained. These all expressed  $\alpha_1$ AT in milk at 13–16

g/liter in their first and subsequent three lactations. Similar levels of expression were obtained in the two subsequent generations and, interestingly, a homozygous female generated by breeding within the line expressed 37 g/liter of  $\alpha_1$ AT; more than double the level of the heterozygous animals (53, 57).

Studies such as these demonstrate the feasibility of generating producer populations that stably transmit and express transgenes. The timelines for the generation of producer populations are, however, quite long. Given that it will be semen from the second generation that is used for breeding (to obviate problems of transmission or genetic instability) then, in sheep and goats it will be a minimum of 3 years before a lactating producer flock can be established; in cattle it will be more than 5 1/2 years before producers are on the ground. In the longer term cloning by nuclear transfer (14–16), particularly if it can be accomplished from expressing adults could have a significant impact on reducing these timelines.

During the establishment of the producer populations detailed characterization of the transgenic locus and the transgene product can be carried out. The animals must of course be housed in quarantined facilities and their health status continuously and individually monitored and a complete history of each individual maintained.

*Purification.* Because of the immunological consequences of administering animal proteins to humans, therapeutic products from the milk of transgenic animals must be purified to a very high degree. Furthermore the product must be pathogen free. The very high concentrations of foreign protein that can be achieved by transgenic approaches facilitates this process. PPL Therapeutics, a company set up to develop this technology have developed a purification process for  $\alpha_1$ AT from transgenic sheep milk involving a combination of skimming, filtration, viral inactivation and chromatography steps. Validation of this process shows that the product produced has up to 23 logs of clearance of viral or prion proteins, should any be present (53). A Pilot Production Plant which processes hundreds of liters of sheep milk a week has been built and this currently produces more than 300 g of GMP grade  $\alpha_1$ AT per week.

*Clinical Trials.* Extensive clinical trials are required for any new therapeutic products destined for human administration so that they can be guaranteed to be both safe and efficacious. Developed countries have regulatory authorities with statutory powers to ensure that new products are tested rigorously in both

preclinical and clinical trials before they can be used. Preclinical tests include biochemical, toxicity and pharmacokinetic properties as well as detailed information on the source and means of production. For protein products derived from the milk of transgenic animals this may include data on the complete sequence of the transgene, structure of the transgenic locus, specificity of transgene expression and the detailed genetic and health histories of the producer lines. These data are reviewed by the relevant authorities as a prelude to consent for the evaluation of the product in humans in clinical trials. Phase I trials are carried out on a small number of healthy volunteers and serve only to determine whether there are any adverse effects. If the phase I trial is successful then phase II trials are initiated to further evaluate safety and efficacy in patient groups and controls. Finally phase III trial are carried out in much larger patient groups and controls to fully evaluate the proposed use of the product. Only when these trials have been successfully completed will a license be issued allowing the manufacturer to sell the product commercially and, hopefully, recoup his investment.

Engaging in clinical trials is a very expensive procedure that invariably costs substantially more than all the other research and development costs put together and carries no guarantee of success. Presently there are two human protein products derived from the milk of transgenic animals that are negotiating this process. Genzyme Transgenics Ltd. is developing antithrombin III produced in the milk of transgenic goats for the treatment of clotting disorders (54) and PPL Therapeutics Ltd. is developing  $\alpha_1$ AT as a treatment to ameliorate lung degeneration in cystic fibrosis (55). Both products successfully completed phase I and are now in phase II trials, so it will be sometime before these products reach the shelf.

## CONCLUSIONS

It is just over 10 years ago that the idea of producing human proteins in the milk of transgenic livestock was conceived and the first experimental approaches initiated (2, 4, 5). In the intervening years substantial progress has been made. Many different human proteins have been targeted to the mammary gland in the mouse as well as in producer animals such as sheep or goats. In some cases extremely high levels of protein production have been achieved, far exceeding those obtainable by conventional batch cell fermentation,

e.g., one transgenic ewe produced more than 30 g/liter  $\alpha_1$ AT in her milk (25). This demonstration of the mammary gland's capacity to produce high levels of a recombinant protein was undoubtedly a milestone in the development of the technology. A key question from the outset was the gland's ability to carry out the necessary posttranslational modifications. By and large, it has risen to the task. Thus  $\alpha_1$ AT produced in transgenic mice or sheep is quite similar (although not identical) to its human plasma derived counterpart (47, 53). However, the glycan structures synthesized by the mammary gland may not always be appropriate as evidenced by the branched mannose chains seen in  $\gamma$ -interferon made in mouse milk (44) and, more seriously, the N-acetylgalactosamine residues observed in htPA produced in goats and cows (45, 46). The mammary gland also seems capable of carrying out complex processing such as  $\gamma$ -carboxylation and proteolytic maturation and the general impression is that it can carry these processes as efficiently, if not more so, than cells in culture. Again the efficiency of these processes may vary between species and target proteins making it important to evaluate carefully each recombinant protein produced and, if necessary, even consider augmenting the posttranslational processes themselves (52). In some cases protein production in the mammary gland has far exceeded our initial expectations. Certainly the demonstration that it can be used to produce high levels of complex multichain proteins such as fibrinogen which are fully active is a tour-de-force. Finally, the fact that two products are already in phase II clinical trials is a testament to the technology and those who have invested the time, effort and money during the last decade.

Notwithstanding these successes, there are certain aspects of the technology that have lagged behind. The fact that up until very recently the means and efficiency of generating transgenic livestock remained unchanged since the first reports of transgenic livestock (5, 7) has been disappointing. Secondly, despite the fact that our basic knowledge of the control of gene expression in the mammary gland has advanced substantially (e.g., see Ref. 58) this knowledge has been of little practical use when it comes to improving transgene expression. Other than using functionally well defined promoter segments and, where possible, including introns there are no robust rules for transgene design and the generation of high expressing lines is still rather a "hit and miss" procedure. Additional strategies have been tried with some success (29) but it remains a moot point

whether there can be a generic solution to improving transgene expression when variables such as the site and structure of the integration locus are outside the investigator's control. Adopting a cell based approach to transgenesis may solve some of these problems. Schneike *et al.* (15) have already shown that nuclear transfer from fibroblasts can be used as a route for transgenesis and, even at this early stage, appears to be more efficient than conventional pronuclear injection in terms of the number of animals required. In the future cloning of transgenic founders may be used to generate and expand producer populations and this could have a very positive impact in terms of cost and timescale. Establishment of gene targeting will enable transgenes to be inserted in a precise manner at defined sites and the expectation is that this may yield more reliably expressing animals. At this time, however, it is not yet clear which sites in the genome should be targeted and, for example, whether the same site would be suitable for different transgenes. Nevertheless, the path ahead is perhaps clearer than it was a year ago and there are evident opportunities to develop and improve this approach for the production of human proteins in livestock.

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## Biotechnology for the production of modified and innovative animal products: transgenic livestock bioreactors

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### Abstract

The ability to modify mammary gland function through genetic engineering provides an opportunity to investigate mechanism of breast cancer, enhance the nutritional quality of milk and synthesize compounds designed as medicine. It is argued that mammary glands are an ideal site for producing complex bioactive proteins that can be harvested and purified in a cost effective manner. The objective of the emerging 'gene pharming' industry is to produce pharmaceuticals for treating human diseases. Consequently, during the past decade, approximately a dozen companies many of them new ventures, have focused their attention on capturing an estimated 3 billion USD annual market for transgenic bioreactor-produced pharmaceuticals. Several products produced in this way are currently in various phases of human clinical trials. The potential profitability of 'gene pharming' looks very promising. © 1999 Elsevier Science B.V. All rights reserved.

**Keywords:** Biotechnology; Mammary gland; Bioactive proteins; Animal diseases

### 1. Introduction

In 1987, scientists at NIH, in collaboration with colleagues at Integrated Genetics, first demonstrated the feasibility of producing a pharmaceutical in the milk of transgenic animals (Gordon et al., 1987). Within five years of that report a new venture-capital-sponsored industry was formed. The remarkable speed with which transgenic animal bioreactor technology moved from laboratories to industry attests to the perceived potential value of this approach for producing pharmaceuticals. However, given the time it takes to put a new drug on the market, it is not surprising that no transgenic-animal-

derived products are as yet in physicians' hands. At least three potential products,  $\alpha$ -1-antitrypsin, antithrombin III and lactoferrin are currently being tested in human clinical trials (Fig. 1).

#### 1.1. Genetic engineering of mammary glands for agriculture

Commercial interests have fueled research on modifying the genetic control of mammary glands for the purpose of producing pharmaceutical proteins in milk. However, transgenic technology also offers the opportunity to alter the composition of bovine milk destined for the dairy industry. A number of excellent reviews have been written on the topic (Richardson, 1985; Wilmut et al., 1990; Yom and Bremel, 1993; Houdebine, 1994; Maga and Murray, 1995). These reviewers and others have suggested

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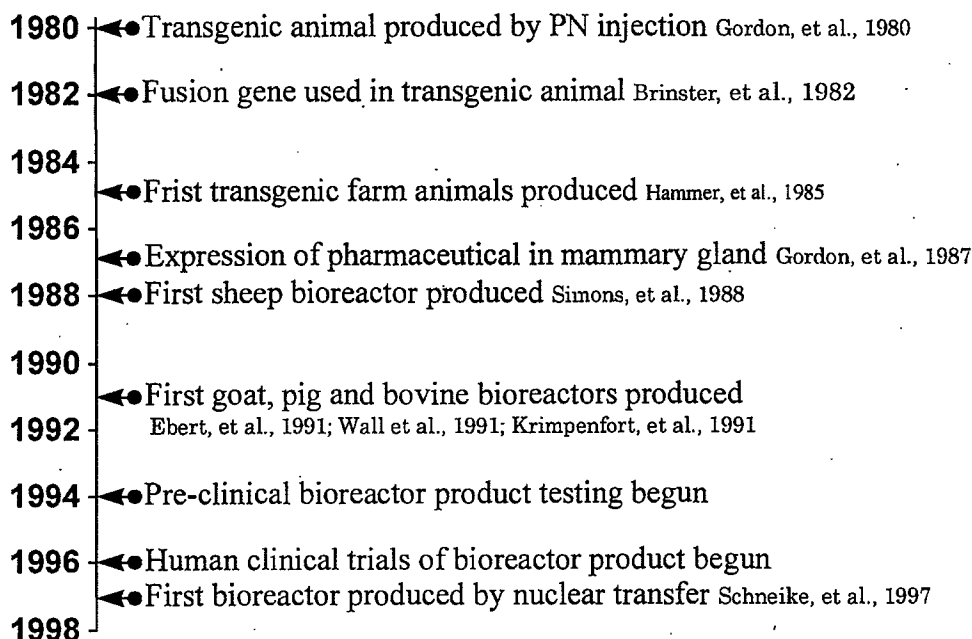


Fig. 1. Milestones in transgenic animal bioreactor technology. Gordon et al. (1980); Brinster et al. (1982); Hammer et al. (1985); Gordon et al. (1987); Simons et al. (1988); Ebert et al. (1991); Wall et al. (1991) Krimpenfort et al. (1991); Schneike et al. (1997).

strategies for changing milk composition to enhance cheese yield, to reduce the energetic cost of milk production and to reduce the microbial load in milk. One of the most ambitious schemes proposed is to 'humanize' bovine milk, by eliminating some bovine milk proteins and replacing others with coding sequences derived from human genes. The resulting milk, which would more closely resemble human breast milk, would be used to supplement or replace infant formula. However, though there have been numerous reviews written, there have been no transgenic livestock produced to test the feasibility of improving milk quality. This is probably because commercial enterprises are choosing to first focus on high unit value commodities and because the high cost of producing transgenic livestock makes it difficult for academic institutions to conduct such studies.

### 1.2. Time requirements for transgenic animal projects

By far, the greatest resource expended in transgenic livestock projects is time. This is clearly

amplified in projects where the transgene is designed to express only in the lactating mammary gland. In such projects, the time from micro-injection to evaluation of milk is equal to twice the gestation length of the target species, plus the time from birth to puberty. Once a desirable line of transgenic animals has been identified, the next step would be to obtain semen in order to generate a production herd. The time required to achieve these milestones using standard techniques is presented in Fig. 2 for

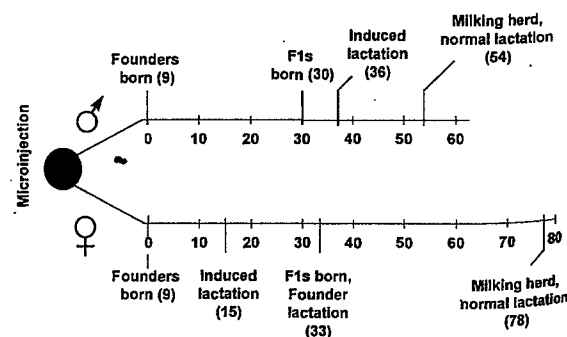


Fig. 2. Time required to produce a herd of lactating transgenic cattle (months).

Table 1  
Comparison of milk production and bioreactor output from livestock species

| Species          | Time to 1st lactation <sup>a</sup><br>(months) | Lactation length<br>(days) | Milk per day<br>(L) | Milk volume<br>(L/lactation) | Drug output<br>per lactation <sup>b</sup> |
|------------------|--|----------------------------|---------------------|------------------------------|---|
| Pig <sup>c</sup> | 17–28  | 50–60                      | 5–10                | 250–500                      | 0.3–0.5                                   |
| Sheep            | 18–31  | 100–200                    | 0.4–2               | 100–300                      | 0.1–0.3                                   |
| Goat             | 18–31  | 200–300                    | 2–4                 | 600–800                      | 0.6–0.8                                   |
| Cattle           | 33–36  | 300                        | 20–30               | 8000                         | 8.0                                       |

<sup>a</sup> First lactation in female founder or daughter of male founder.

<sup>b</sup> Assumes bioreactor product concentration of 1 g/l.

Adapted from presentation by John Curling, John Curling Consulting AB 'Challenges and possible solutions in the purification of transgenic biopharmaceuticals.' Third Annual International Symposium on Producing the Next Generation of Transgenic Therapeutics. February 5–6, 1997, West Palm Beach, FL, USA

cattle and in Table 1 for other farm animals. Clearly, there is a need for development of strategies to shorten this time commitment.

A number of strategies have been demonstrated or proposed to reduce the time required to identify and evaluate transgenic dairy animals as well as the time required to expand successful lines (Bondioli and Wall, 1998). For example, identification of animals expressing a lactation-specific transgene has been performed on milk obtained by artificial induction of lactation in female and male goats (Ebert et al., 1994). More recently, expression of a fusion gene composed of the bovine  $\alpha$ S1-casein promoter driving the human growth hormone gene was detected in both male and female rat mammary biopsies obtained 24h after birth (Hirabayashi et al., 1996). If such a strategy were proven to be reflective of adult milk production, then semen from a transgenic bull, collected at approximately 15 months of age (two years following microinjection) could be used to generate a herd producing genetically modified milk in approximately three years – about five years from the time of microinjection. However, even with improved evaluation methods and the possible increase in efficiency that nuclear transfer may offer (Schnieke et al., 1997), the time and financial investment in transgenic cattle projects must be carefully evaluated in terms of economic return on investment.

## 2. Transgenic animal bioreactors

A number of companies have been formed within the last decade specifically to exploit transgenic

technology. This new industry has been created because of the perception that production of pharmaceuticals in transgenic animals will be more cost effective than production by more conventional means. The world market for the first generation of products identified as appropriate for this technology exceeds 3 billion USD a year (Table 2). It is noteworthy that all of the products listed in Table 1 are currently derived from human blood, which may account for the fact that the American Red Cross Blood Derivatives Laboratory is a central player in the field.

### 2.1. Are there better bioreactors?

Almost any living organism, or part thereof, that can be 'domesticated' could serve as a bioreactor. Bacteria, yeast, insect cells, mammalian cells in culture, plants, and chicken eggs are all potential competing production systems. Each system has specific advantages and disadvantages. In general, prokaryotic systems and plants can be rapidly genetically engineered and propagated at relatively low cost; however many of these organisms lack the mechanisms or possess the wrong machinery to perform some of the critical post-translational modifications (e.g. signal peptide cleavage, glycosylation, amidation, acetylation, carboxylation, and phosphorylation) required by complex mammalian proteins (Houdebine, 1994). Baculovirus-insect cell expression systems (Luckow and Summers, 1988) and stable transfected mammalian cells have the capacity to perform authentic post-translational modifications, but yields in those systems are often an order of magnitude lower than those already

Table 2

Estimated annual world-wide requirements and costs of potential bioreactor products<sup>a</sup>

| Item                                 | Pharmaceutical      |                   |           |                     |            |                          |
|--------------------------------------|---------------------|-------------------|-----------|---------------------|------------|--------------------------|
|                                      | F-VIII <sup>b</sup> | F-IX <sup>c</sup> | Protein C | AT III <sup>d</sup> | Fibrinogen | Albumin <sup>e</sup>     |
| Estimated quantity needed            | 304 g               | 4 kg              | 10 kg     | 21 kg               | 150 kg     | 315 × 10 <sup>3</sup> kg |
| Current cost per gram (\$)           | 2,900,000           | 40,000            | 10,000    | 7,000               | 1,000      | 3.56                     |
| Annual market (10 <sup>6</sup> × \$) | 882                 | 160               | 100       | 150                 | 150        | 1,120                    |

<sup>a</sup> Information from William Drohan and Henryk Lubon, American Red Cross, (Paleyanda et al., 1991; Lee and de Boer, 1994).<sup>b</sup> Blood coagulating Factor VIII.<sup>c</sup> Blood coagulating Factor IX.<sup>d</sup> AntiThrombin III.<sup>e</sup> Human serum albumin.

achieved in transgenic animal bioreactors. The perceived utility of the mammary gland may, in part, account for the considerable attention that has been focused on investigating transgene expression in mammary glands. Table 3 presents a partial list of transgenic mammary gland projects. Though Table 3 includes studies designed to address both basic and applied aspects of expressing additional protein in the mammary gland, the bulk of the research literature is focused on biomedical rather than agricultural applications. Examining the references in Table 3 it is interesting to note that most of the animal bioreactor organizations have distinguished themselves by obtaining intellectual property protection by either applying for patents or exclusively licensing specific mammary gland regulatory elements (e.g. American Red Cross, mouse Whey Acidic Protein; Genzyme Transgenics, goat J-casein; Pharming, BV, bovine  $\alpha_{s1}$ -casein; PPL Therapeutics, sheep J-lactoglobulin). As intended, patent protection gives organizations proprietary rights to mammary gland specific promoters. However, as most of the mammary specific regulatory elements become protected new bioreactor organizations have little choice but to license promoters or challenge existing patents.

## 2.2. Advantages of the transgenic mammary gland

The mammary gland is a prodigious production system, capable of generating between 23 g (dairy cattle) and 205 g (rat) of protein per kg of body weight during peak lactation (Ofstedal, 1984). Milk is

clearly the least complicated bodily fluid to collect, especially from ruminants. Milking of pigs can even be mechanized (Grun et al., 1993). The ample production capacity of the mammary gland coupled with the relative ease of harvesting milk in a non-invasive manner recommends the mammary gland as the organ of choice for producing pharmaceutical products in animals.

Another often cited advantage of producing biologically active products in the mammary gland is the isolation of the mammary gland from the circulatory system. It is argued that bioreactor animals would be protected from the potentially untoward effects of biologically active compounds because those compounds would be sequestered in the mammary gland and therefore unavailable to the circulatory system. However, endogenous milk proteins are indeed found in the circulation in cattle, especially during late gestation and at the time of parturition (McFadden et al., 1987), and transgenes and milk protein genes are transiently expressed during estrus, even in virgin mice (Robinson et al., 1995). Therefore, to safeguard bioreactor animals, it may be appropriate to consider designing gene constructs in such a way that their product is converted to an active form after it is isolated from milk.

A potential hurdle in the success of mammary gland bioreactors lies in the ability of alveolar epithelium to provide appropriate post-translational modifications such as cleavage of pro- or signal peptides, N- or O-glycosylation, and  $\gamma$ -carboxylation. It can be assumed that lack of faithful post-transla-

Table 3

A partial list of transgenic animal studies in which a milk protein promoter was used to direct expression of milk and pharmaceutical proteins to the mammary gland

| Coding sequence             |                 | Transgenic species | Promoter region        |            | Protein in milk (ml <sup>-1</sup> ) | Ref  |
|-----------------------------|-----------------|--------------------|------------------------|------------|-------------------------------------|--|
| Gene                        | Source          |                    | Gene                   | Source     |                                     |  |
| $\alpha_1$ antitrypsin      | mouse           | mice               | WAP                    | rabbit     | mg                                  | Massoud et al., 1991   |
| $\alpha_1$ antitrypsin      | human           | mice               | $\beta$ -lactoglobulin | ovine      | mg                                  | Archibald et al., 1990   |
| $\alpha_1$ antitrypsin      | human           | sheep              | $\beta$ -lactoglobulin | ovine      | mg                                  | Soulier et al., 1992   |
| $\alpha$ -glucosidase       | human           | mice               | $\alpha_{s1}$ -casein  | bovine     | $\mu$ g                             | Bijvoet et al., 1996   |
| $\alpha$ -lactalbumin       | bovine          | mice               | $\alpha$ -lactalbumin  | bovine     | mg                                  | Vilotte et al., 1989; Bleck and Bremel, 1994; Soulier et al., 1992 |
| $\alpha$ -lactalbumin       | goat            | mice               | $\alpha$ -lactalbumin  | goat       | mg                                  | Maschio et al., 1991   |
| $\alpha$ -lactalbumin       | guinea-pig      | mice               | $\alpha$ -lactalbumin  | guinea-pig | ND                                  | Clarke et al., 1994  |
| $\alpha$ -lactalbumin       | human           | rats               | $\alpha$ -lactalbumin  | human      | mg                                  | Fujiwara et al., 1997  |
| $\alpha_{s1}$ -casein       | bovine          | mice               | $\alpha_{s1}$ -casein  | bovine     | ND                                  | Rijnkels et al., 1995  |
| Anti-CD6 antibodies         | mouse/<br>human | mice               | WAP                    | rabbit     | ND                                  | Limonta et al., 1995   |
| Antithrombin III            | human           | goat               | J-casein               | goat       | mg                                  | Meade and Groet, 1997  |
| $\beta$ -casein             | bovine          | mice               | $\beta$ -casein        | bovine     | mg                                  | Bleck et al., 1995   |
| $\beta$ -casein             | bovine          | mice               | $\alpha$ -lactalbumin  | bovine     | mg                                  | Persuy et al., 1992  |
| $\beta$ -casein             | goat            | mice               | $\beta$ -casein        | goat       | mg                                  | Roberts et al., 1992; Lee et al., 1988                             |
| $\beta$ -casein             | rat             | mice               | $\beta$ -casein        | rat        | mg                                  | Schellander and Peli, 1992   |
| $\beta$ -interferon         | human           | mice               | WAP                    | mouse      | nd                                  | Simons et al., 1987  |
| $\beta$ -lactoglobulin      | ovine           | mice               | $\beta$ -lactoglobulin | ovine      | mg                                  | Dobrovolsky et al., 1993   |
| $\gamma$ -interferon        | human           | mice               | $\beta$ -lactoglobulin | ovine      | ng                                  | Bleck et al., 1996   |
| $\kappa$ -casein            | bovine          | mice               | $\beta$ -casein        | goat       | mg                                  | Persuy et al., 1995  |
| $\kappa$ -casein            | goat            | mice               | $\beta$ -casein        | goat       | mg                                  | DiTullio et al., 1992  |
| CFTR                        | human           | mice               | $\beta$ -casein        | goat       | $\mu$ g                             | Yull et al., 1995  |
| EPO                         | human           | mice               | $\beta$ -lactoglobulin | bovine     | mg                                  | Korhonen et al., 1997  |
| EPO                         | human           | rabbis             | $\beta$ -lactoglobulin | bovine     | mg                                  | Korhonen et al., 1997  |
| Factor VIII                 | human           | sheep              | $\beta$ -lactoglobulin | ovine      | nd                                  | Platenburg et al., 1994  |
| Factor IX                   | human           | mice               | $\beta$ -lactoglobulin | ovine      | $\mu$ g                             | Krimpenfort et al., 1991   |
| Factor IX                   | human           | sheep              | $\beta$ -lactoglobulin | ovine      | nd                                  | Clark, 1989  |
| Fibrinogen                  | human           | mice               | WAP                    | mouse      | mg                                  | Butler et al., 1997  |
| FSH                         | bovine          | mice               | J-casein               | rat        | $\mu$ g                             | Greenberg et al., 1991   |
| GM-CSF                      | human           | mice               | $\alpha_{s1}$ -casein  | bovine     | $\mu$ g                             | Uusi-Oukari et al., 1997   |
| Growth hormone              | bovine          | mice               | WAP                    | rat        | mg                                  | Thepot et al., 1995  |
| Growth hormone              | human           | mice               | J-casein               | rat        | mg                                  | Lee et al., 1996   |
| Hepatitis B surface antigen | human           | goat               | $\alpha_{s1}$ -casein  | bovine     | ND                                  | Zhang et al., 1997   |
| IGF-I                       | human           | rabbis             | $\alpha_{s1}$ -casein  | bovine     | mg                                  | Brem et al., 1994  |
| Interleukin-2               | human           | rabbis             | $\beta$ -casein        | rabbit     | ng                                  | Buhler et al., 1990  |
| Lactoferrin                 | human           | mice               | $\alpha_{s1}$ -casein  | bovine     | mg                                  | Platenburg et al., 1994  |
| Lactoferrin                 | human           | cattle             | $\alpha_{s1}$ -casein  | bovine     | ND                                  | Krimpenfort et al., 1991   |
| Lysozyme                    | human           | mice               | $\alpha_{s1}$ -casein  | bovine     | $\mu$ g                             | Maga et al., 1994  |
| Protein C                   | human           | mice               | WAP                    | mouse      | ng                                  | Velander et al., 1992a   |
| Protein C                   | human           | pigs               | WAP                    | mouse      | mg                                  | Velander et al., 1992b   |
| Serum albumin               | human           | mice               | $\beta$ -lactoglobulin | ovine      | mg                                  | Shani et al., 1992   |
| Superoxide dismutase        | human           | mice               | $\beta$ -lactoglobulin | ovine      | ng                                  | Hansson et al., 1994   |
| Superoxide dismutase        | human           | mice               | WAP                    | mouse      | mg                                  | Hansson et al., 1994   |
| Surfactant protein B        | human           | mice               | WAP                    | rat        | ug                                  | Yarus et al., 1997   |
| TAP                         | human           | mice               | WAP                    | rat        | ug                                  | Yarus et al., 1996   |
| t-PA                        | human           | mice               | WAP                    | mouse      | ng                                  | Gordon et al., 1987  |
| t-PA                        | human           | mice               | $\alpha_{s1}$ -casein  | bovine     | $\mu$ g                             | Riego et al., 1993   |
| t-PA                        | human           | rabbis             | $\alpha_{s1}$ -casein  | bovine     | $\mu$ g                             | Riego et al., 1993   |
| t-PA                        | human           | goats              | WAP                    | mouse      | $\mu$ g                             | Ebert et al., 1991   |
| Trophoblastin               | sheep           | mice               | $\alpha$ -lactalbumin  | bovine     | $\mu$ g                             | Stinnakre et al., 1991   |
| Urokinase                   | human           | mice               | $\alpha_{s1}$ -casein  | bovine     | mg                                  | Meade et al., 1990   |
| WAP                         | mouse           | mice               | WAP                    | mouse      | mg                                  | Burdon et al., 1991  |
| WAP                         | rat             | mice               | WAP                    | rat        | mg                                  | Dale et al., 1992  |
| WAP                         | mouse           | pigs               | WAP                    | mouse      | mg                                  | Shamay et al., 1991; Wall et al., 1991                             |
| WAP                         | mouse           | sheep              | WAP                    | mouse      | $\mu$ g                             | Wall et al., 1996  |

tional modification of a protein will affect its biological activity. Furthermore, it is well known that carbohydrate residues of glycoproteins can serve as antigens. Those residues can also influence secretion of a protein and affect a protein's half-life in the circulation (James et al., 1995).

It has recently been shown that when transgenes for interferon- $\gamma$  were expressed in Chinese hamster ovary cells, baculovirus-infected Sf9 insect cells, and mammary glands of transgenic mice, N-glycosylation patterns (sites glycosylated) and composition of the sugar residues differed significantly (James et al., 1995). It has also been demonstrated that glycosylation patterns of Protein C produced in the mammary glands of mice differ from Protein C isolated from human serum (Drohan et al., 1994). The glycosylation patterns of Protein C isolated from transgenic pig milk also differed from that observed in human serum (Morcol et al., 1994). Furthermore, the Protein C carbohydrate patterns in mouse milk and pig milk differed. No additional data are available to assess the significance of these observations on the transgenic bioreactor approach to producing pharmaceuticals. However, at least one prominent laboratory is beginning to address the issue by creating transgenic mice containing two mammary gland-directed transgenes, one for the protein of interest, the other to increase the gland's post-translational modification (Drews et al., 1995).

### *2.3. Does urine make good dollars and sense*

The transgenic animal bioreactor industry has focused primarily on directing expression of their products to the milk, though at least one organization has explored the possibility of isolating products from blood (Swanson et al., 1992). Using a 3.6-kb promoter of mouse uroplakin II gene, we have generated transgenic mice that express human growth hormone (hGH) in their bladder epithelium, resulting in its secretion into the urine at 100–500 ng/ml (Kerr et al., 1998). The levels of urine hGH concentration remained relatively constant for eight months and were similar for males and females. Secreting substances into urine from the bladder offers some of the same advantages as the mammary gland: straightforward, noninvasive collection of the product. In addition, the bladder bioreactor provides

the main advantage of blood based systems: the ability to harvest products shortly after birth from both sexes. However, the concentration of product in the urine is only about 10% of that which can be generated in mammary gland bioreactors in the three lines studied. The low urinary concentration of product might, however, be offset by the relative ease of isolating drugs from urine when compared to the more extensive purification schemes required for complex fluids such as blood and milk. Further, research will have to be conducted to assess the value of this approach.

### *2.4. Production capacity of transgenic mammary glands*

It is difficult to predict transgene production capacity with any certainty. However, from the growing list of transgenic animal studies in which a milk protein promoter has been used to direct expression of pharmaceutical or milk protein (Table 3) into milk, it seems reasonable to expect production levels of at least 1 mg/ml. Unfortunately, there have been too few comparative transgenic animal studies done to make firm recommendations on transgene design. Evaluating transgene design criteria is especially difficult because of the wide variation in expression that is generally observed between lines containing the same transgene. Thus, even a relatively simple comparison between constructs is a formidable task.

The bovine is the target species for transgenic projects aimed at modifying milk for the dairy industry. For 'pharming' projects, the target species will be largely determined by how much product is needed. Assuming a production level of 1 mg/ml, one can calculate the number of animals that would be required to produce some of the proposed pharmaceutical bioreactor products. The results of those calculations are presented in Table 4. On first inspection, it seems unreasonable to think an organization would consider generating the more than 100,000 rabbits necessary to produce 150 kg of fibrinogen. The labor required to maintain and milk those animals would be enormous, especially in light of the fact that 17 cows might be capable of producing all of the required fibrinogen to satisfy current world needs. However, the required number

Table 4

Estimated number of transgenic animals needed to satisfy the annual world market for selected pharmaceutical<sup>a</sup>

| Species             | Pharmaceutical      |                   |                  |                     |                   |                      |
|---------------------|---------------------|-------------------|------------------|---------------------|-------------------|----------------------|
|                     | F-VIII <sup>b</sup> | F-IX <sup>c</sup> | Protein C        | AT III <sup>d</sup> | Fibrinogen        | Albumin <sup>e</sup> |
| Rat                 | 345                 | 4,454             | $11 \times 10^3$ | $24 \times 10^3$    | $171 \times 10^3$ | $358 \times 10^6$    |
| Rabbit              | 217                 | 2,857             | 7,143            | 15,000              | $107 \times 10^3$ | $225 \times 10^6$    |
| Pig                 | 2                   | 15                | 38               | 81                  | 577               | $1212 \times 10^3$   |
| Goat                | 1                   | 3                 | 6                | 12                  | 83                | 175,000              |
| Sheep               | 1                   | 1                 | 3                | 6                   | 45                | 93,000               |
| Cattle <sup>f</sup> | 1                   | 1                 | 2                | 3                   | 17                | 35,000               |

<sup>a</sup> Based on estimated quantity needed (Table 1) and assuming a transgene protein production capacity of 1 g/l. capacity.<sup>b</sup> Blood coagulating Factor VIII.<sup>c</sup> Blood coagulating Factor IX.<sup>d</sup> AntiThrombin III.<sup>e</sup> Human serum albumin.<sup>f</sup> Assumes 1 g/l production of transgenic protein in cattle.

of rabbits could be produced in a little more than a year by using homozygous males and artificial insemination, whereas, it would take longer than four years to produce the 17 cows (Table 5). This is an exaggerated comparison, but it points out the need to consider generation interval as well as production capacity when choosing a species for a bioreactor project.

To further put the values in Table 4 in perspective, the small herd of pigs or couple of cows required to satisfy the market needs for Protein C could substitute for five million liters of plasma. The sizeable herd of 35,000 cows needed to supply the more than 400 tons of human serum albumin could obviate the need for collecting 16 million liters of blood.

Though the data presented in Tables 4 and 5 are admittedly based on preliminary findings, they bode well for the practical ability to genetically engineer mammary glands to produce commercially useful concentrations of foreign proteins. But from a bio-

logical perspective, transgene protein production efficiency values are low for all species with the possible exception of sheep. Are the relatively modest concentrations of transgene proteins observed the result of inept transgene design or are mammary glands already producing close to their maximum capacity? Will milk protein genes have to be 'knocked out' to provide additional capacity for transgene proteins? Will higher transgene product production levels be disruptive to mammary gland function as they were in one study (Bleck et al., 1995)? These questions can not be answered at this time.

### 3. Current animal bioreactor industry concerns

As the leaders in the animal bioreactor industry move from their initial R and D phase, which involved creating gene constructs and producing transgenic animals, into drug production, some of the originally identified concerns persist. Issues such as producing biological active molecules that are faithfully modified after translation and mimic the glycosylation patterns decorating the homologue isolated from humans is still of concern. Also, decreasing development time and costs are still receiving attention. However, if presentations given at IBC's Third Annual International Symposium on Producing the Next Generation of Transgenic Therapeutics held in West Palm Beach, Florida, February 5th and 6th, 1997 are indicative, the industry is very

Table 5

Estimate of the shortest number of months required to produce transgenic animals needed to satisfy the annual world market for blood coagulating Factor IX and Fibrinogen<sup>a</sup>

| Species | Pharmaceutical |            |
|---------|----------------|------------|
|         | Factor IX      | Fibrinogen |
| Rabbit  | 15             | 27         |
| Sheep   | 18             | 26         |
| Pig     | 37             | 59         |
| Cattle  | 33             | 54         |

<sup>a</sup> Based on number of animals needed (Tables 3 and 4).

much focused on developing plants for producing product, building production facilities and purifying large amounts of product for clinical trials.

Speakers at the West Palm Beach meeting from Genzyme Transgenics, Pharming, BV and PPL Therapeutics generally agreed that the major issues currently facing the industry include identifying lines of transgenic animals producing biologically active transgene product at high concentrations, developing breeding strategies to create a production herd, and creating a well characterized purification scheme that yields high purity, pathogen free product. The processing system should be easily scalable, reproducible and achievable at low cost.

It was only a few years ago that the bioreactor industry was focused on the costs of producing the transgenic animals. That seemed an appropriate concern given the estimated costs of producing founder animals carrying a functional transgene range from 30,000 USD for a transgenic pig to over

300,000 USD for a transgenic cow (Wall et al., 1992). However, the industry is discovering that the costs of producing founder animals are not the most expensive aspect of the manufacturing process. The costs of making the bioreactor animals, building the facilities to house them and building the processing plant are fixed in the sense that they only have to be incurred once per product. Whereas the costs of quality control and consumables used for purification are more or less continuous. The costs of purification media is of little concern for most products of high market value such as erythropoietin (EPO) and Factor VIII (Fig. 3) which have both high market value and high unit price (dollars/gram). The industry clearly recognized the merit of targeting high market value products. As can be seen in Fig. 3 all of the proposed first generation bioreactor products are clustered in the top left of Fig. 3, with one exception. Human serum albumin (HSA) has an estimated unit price of only about 3.50 USD, but the amount of

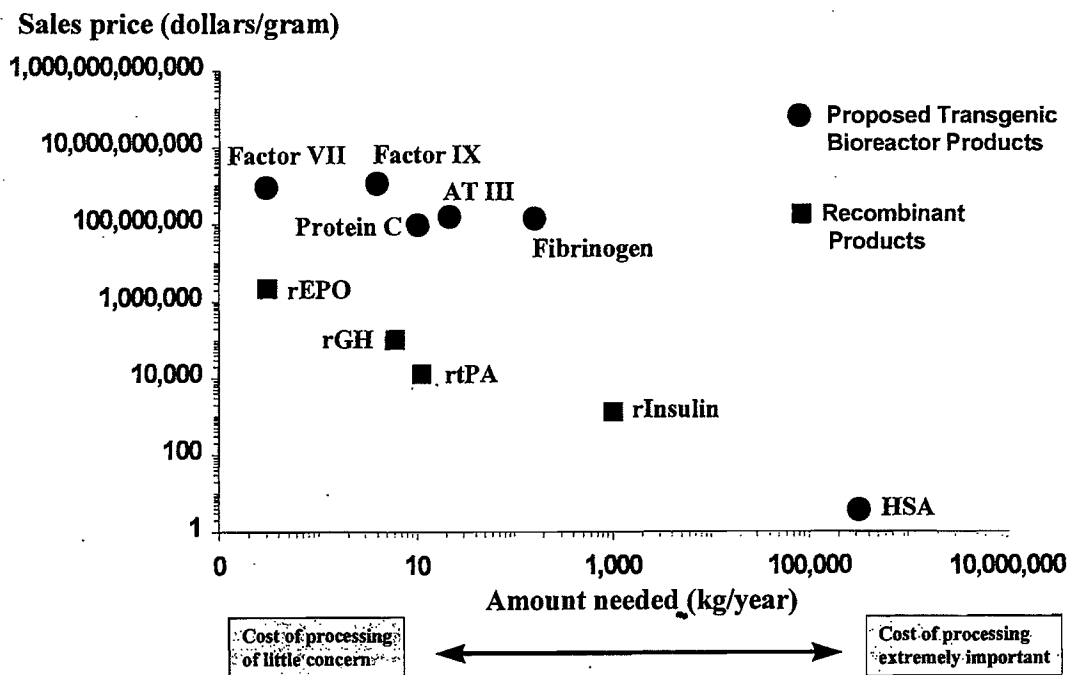


Fig. 3. Estimated selling price and amount needed of recombinantly produced and proposed bioreactor products. AT III, antithrombin III; HAS, human serum albumin; rEPO, recombinant erythropoietin; rGH, recombinant growth hormone; rtPA, recombinant tissue plasminogen activator; rInsulin, recombinant insulin. Adapted from the presentation by John Curling, John Curling Consulting AB. Challenges and possible solutions in the purification of transgenic biopharmaceuticals. Third Annual International Symposium on Producing the Next Generation of Transgenic Therapeutics. February 5–6, 1997, West Palm Beach, FL, USA., and Wall et al., 1996.

HSA consumed is said to exceed 400 tons annually. Thus the market value of HSA is more than a billion dollars (US) per year (Table 1). Nevertheless, for a manufacturing process to be profitable HSA will have to be produced inexpensively.

The pharmaceuticals depicted in Fig. 3 are only the first generation of drugs the bioreactor industry targeted. As more companies enter the field and as the original entrants gain a better understanding of the utility of this approach for manufacturing complex molecules, the industry has expanded the list of products that they are considering as potential candidates. A partial list of the second generation proteins that have been mentioned are listed in Table 6.

It appears that industry leaders are in basic agreement as to the approach for establishing their production herds. Most organizations have chosen to produce several lines of transgenic animals from which one line is selected to serve as foundation stock for generation of the production herd. It is necessary to produce multiple transgenic lines because each line usually differs in the amount of transgene product they produce. Unfortunately there currently is no way to predict transgene expression levels in mammary gland bioreactors before lactation

(Wall, 1996). Selecting a single 'good' line to serve as foundation stock may be of value to reduce variation in concentration of product reaching the processing plant. Focusing on a single line will definitely reduce the substantial effort required by FDA to fully characterize the site of integration of the transgene in production animals.

Interestingly, issues well known to the dairy industry, are becoming a concern of the bioreactor industry. Developing a consistent year round supply of milk from seasonal breeding animals such as ruminants, obtaining a supply of consistent quality feed, and dealing with changing milk composition during lactation are factors that complicate the purification process. Fluctuations in milk composition require costly adjustments of purification schemes to maximize product yield and minimizing cost of purification. Changing protein and fat concentrations and their ratio make it imperative to have a sophisticated milk quality control program in place to monitor the changes so that purification steps can be appropriately altered.

Dr. John Curling, a purification specialist, pointed out that the animal bioreactor industry benefits from the fact that most modern industrial purification

Table 6  
Examples of second generation animal bioreactor products

| Category          | Examples                     | Organization                |
|-------------------|------------------------------|-----------------------------|
| Antibodies        | BR96                         | Genzyme<br>PPL Therapeutics |
| Food Products     | Caseins                      | Nexia                       |
| Generics          | Growth Hormone               | Nexia                       |
| Hormones          | Calcitonin                   | Genzyme Transgenics         |
| Immunologics      | Interferons                  | Genzyme Transgenics         |
| Nutraceuticals    | I-Lactalbumin variant        | Abbott Laboratories         |
|                   | Bile Salt Stimulate Lipase   | Astra Hassle AB             |
|                   | J-Lactoglobulin knock-out    | Pharming BV                 |
|                   | Fucosyltransferase           | PPL Therapeutics            |
|                   | Lactase                      | Nexia                       |
|                   | Lactoferrin                  |                             |
|                   | Lysozyme                     |                             |
| Peptides          | Insulin                      | Genzyme Transgenics         |
| Plasma Proteins   | I-1-Proteinase Inhibitor     | Genzyme Transgenics         |
| Receptors         | Soluble CD4                  | Genzyme Transgenics         |
| Recombinant       | Tissue Plasminogen Activator | Genzyme Transgenics         |
| Tissue remodeling | Collagen                     | Collagen Corp.              |
|                   | Fibrinogen                   | Pharming BV<br>ZymoGenetics |
| Vaccines          | Malaria                      | Genzyme Transgenics         |

practices where originally developed for the dairy industry. Therefore, theories and equipment for purifying substances from milk do not have to be invented de novo. Purification strategies designed for isolating compounds from milk involve three basic steps. First gross contaminants, such as fat, caseins, lactose, minerals and cells are removed. That is followed by removal of endogenous milk proteins and finally separation of the end product from trace contaminants. As is illustrated in Fig. 4, one of the primary factors that influence the cost of purification is the concentration of the product in the starting material. In the fictitious example shown in Fig. 4, if purification media cost 3000 USD per liter the cost of purified product is reduced by half, from 3 USD/gram to 1.50 USD/gram if the initial concentration of product in the starting material is increased from 10 g/l to 20 g/l. That would seem to argue that lines should be selected for production that produce the highest concentration of product in their milk. However, it appears that even though the mammary gland may have a immense capacity to transcribe genes and translate them into protein, its capacity for post-translational modifications of the proteins may be finite (Drohan et al., 1994; Drews et al., 1995). That is significant since correct post-translational modifications are critical for conferring appropriate biological activity. Furthermore, since one of the major arguments in favor of mammary gland bioreactors, over other drug production systems is the mammary

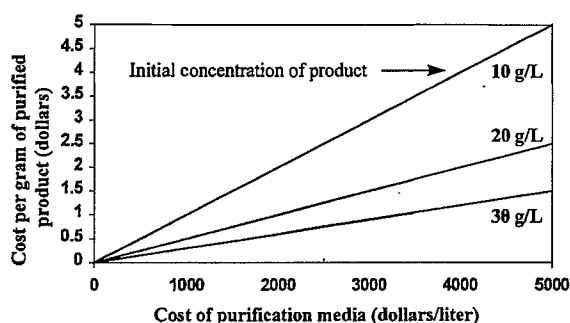


Fig. 4. Influence of initial concentration of product in starting material and cost of purification media on cost of purified product. Adapted from the presentation by John Curling, John Curling Consulting AB. Challenges and possible solutions in the purification of transgenic biopharmaceuticals. Third Annual International Symposium on Producing the Next Generation of Transgenic Therapeutics. February 5–6, 1997, West Palm Beach, FL, USA.

gland's ability to produce correctly processed complex proteins, the industry may have to accept higher costs of purification attendant with lower product concentrations to insure of correctly processed molecules.

As the industry faces the challenges of purification from chemical components of milk they also have to be concerned with removing somatic cells, bacteria and adventitious viruses. At the close of John Curling's talk at Third Annual International Symposium on Producing the Next Generation of Transgenic Therapeutics he pointed out the obvious, 'Each product poses different purification problems' and the not so obvious, 'Purification starts with the choice of bioreactor animal.'

The industry appears fully capable of dealing with the challenges presented by purification and is energized by the prospects of having product on the market within the decade. However, the animal bioreactor industry faces an interesting outside challenge that could have a significant impact on the long term utility of animal bioreactors.

The challenge comes from a rapidly developing sister industry, plant bioreactors. Genetically engineered plants may not be able to compete with animal bioreactors for every therapeutic compound. But where the two approaches can produce the same molecule at equal purity and biological activity, the plant system will be a formidable competitor.

One of the more malleable plants to genetically engineer is tobacco. Because of the increasing efforts by governments to restrict smoking, the tobacco industry is receiving new funding to find alternative uses for tobacco. Some of that money is fueling the development of plant bioreactors. Plants have been engineered to make human glucocerebrosidase (Carol Crammer, CropTech), I-galactosidase, J-globin, K-interferon, NP1 defensin, and I-trichosanthin (Larry Grill, Biosource Technology) and edible vaccines against cholera, norwalk virus, hepatitis and rotavirus (Liz Richter, Boyce Thompson Institute for Plant Research). These examples presented at Third Annual International Symposium on Producing the Next Generation of Transgenic Therapeutics illustrates the potential utility of plants in general and tobacco in the specific.

It remains to be seen how the competition in the plant and animal bioreactor industries will play out.

But it is clear that in the coming decade the medical community will see less expensive therapeutics produced in a variety of non-conventional ways.

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# Transgenic Dairy Cattle: Genetic Engineering on a Large Scale

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## ABSTRACT

Amid the explosion of fundamental knowledge generated from transgenic animal models, a small group of scientists has been producing transgenic livestock with goals of improving animal production efficiency and generating new products. The ability to modify mammary-specific genes provides an opportunity to pursue several distinctly different avenues of research. The objective of the emerging gene "pharming" industry is to produce pharmaceuticals for treating human diseases. It is argued that mammary glands are an ideal site for producing complex bioactive proteins that can be cost effectively harvested and purified. Consequently, during the past decade, approximately a dozen companies have been created to capture the US market for pharmaceuticals produced from transgenic bioreactors estimated at \$3 billion annually. Several products produced in this way are now in human clinical trials. Another research direction, which has been widely discussed but has received less attention in the laboratory, is genetic engineering of the bovine mammary gland to alter the composition of milk destined for human consumption. Proposals include increasing or altering endogenous proteins, decreasing fat, and altering milk composition to resemble that of human milk. Initial studies using transgenic mice to investigate the feasibility of enhancing manufacturing properties of milk have been encouraging. The potential profitability of gene "pharming" seems clear, as do the benefits of transgenic cows producing milk that has been optimized for food products. To take full advantage of enhanced milk, it may be desirable to restructure the method by which dairy producers are compensated. However, the cost of producing functional transgenic cattle will remain a severe limitation to realizing the potential of transgenic cattle until ineffi-

ciencies of transgenic technology are overcome. These inefficiencies include low rates of gene integration, poor embryo survival, and unpredictable transgene behavior.

(**Key words:** transgenic, bioreactor, genetic engineering, milk composition)

**Abbreviation key:** CoA = coenzyme A, hGH = human growth hormone.

## INTRODUCTION

In 1987, Lothar Hennighausen and Heiner Westphal at the National Institutes of Health, in collaboration with Katy Gordon and her colleagues at Integrated Genetics, started a mini-revolution when they reported that a pharmaceutical could be produced in mammary glands of transgenic animals (28). Within 5 yr of their report, a new industry was formed, sponsored with venture capital. The remarkable speed with which transgenic animal bioreactor technology moved from laboratories to industry attests to the perceived potential value of this approach for producing pharmaceuticals. The dairy industry has not embraced transgenic technology with the same enthusiasm as the pharmaceutical industry. In this review, we explain why and try to identify productive areas of research that could provide supporting justification for the dairy industry to consider the transgenic approach as a practical means to enhance the genetic potential of dairy cattle.

## Goals for Genetic Engineering of Mammary Glands

Commercial interests have fueled research on modifying the genetic control of mammary glands for the purpose of producing pharmaceutical proteins in milk. However, this technology also offers the opportunity to alter the composition of bovine milk destined for the dairy industry. A number of excellent reviews (38, 49, 60, 82, 84) have been written on the topic. The authors of those reviews have suggested strategies for changing milk composition to enhance cheese yield, to reduce the energetic cost of milk production,

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TABLE 1. Milestones in transgenic animal bioreactor technology.

| Year | Accomplishment   | Reference        |
|------|--|------------------|
| 1980 | Transgenic animal produced by pronuclear microinjection.                       | (27)             |
| 1982 | Fusion gene introduced into transgenic mice.                                   | (10)             |
| 1987 | Expression of pharmaceutical directed to the mammary gland of transgenic mice. | (28)             |
| 1988 | First transgenic bioreactor farm animals produced (sheep).                     | (69)             |
| 1991 | Mammary gland specific transgene expressed in goats.                           | (22)             |
| 1991 | Mammary gland specific transgene expressed in pigs.                            | (79)             |
| 1991 | Transgenic bull produced with mammary gland specific gene construct.           | (43)             |
| 1994 | Bioreactor products being tested in animal models (preclinical testing).       | ... <sup>1</sup> |

<sup>1</sup>Henryk Lubon (1994, personal communication).

and to reduce the microbial load in milk. One of the most ambitious schemes that has been proposed is to alter the composition of bovine milk so that it resembles human milk by eliminating some bovine milk protein genes and replacing others with coding sequences derived from human genes. The resulting milk, which would more closely resemble human breast milk, would be used to supplement or replace infant formula.

### Economic Considerations

Genetic improvements in livestock will be judged by the agricultural industry solely on total economic merit. Assessing the cost-benefit ratios of a project is a complicated process at best; assessing the economics of projects directed toward solving problems of human disease, which involve moral considerations, adds to the complexity. However, the economics of even those types of projects can and should be evaluated. Cost assessment of the merits of a given strategy for improving livestock traits is more straightforward. With current technology, the cost of producing transgenic cows is so great that very few organizations have been willing to invest in experiments designed to assess the technical feasibility of altering cow's milk to create new food products. Without that information, it may be premature to speculate on other, potentially larger, project costs that may be incurred to alter marketing strategies and processing systems and to meet regulatory requirements for the new animal products.

Time is the greatest resource expended in a transgenic cattle project. Using techniques now considered standard, 7 to 8 yr are needed to produce a milking herd of transgenic dairy cattle. Policy makers should not view this situation as a justification to abandon the development of these technologies but, rather, as an indication that additional research resources should be made available to overcome the impediments that hinder progress.

Before gene modifications relating to the mammary gland are discussed in detail, it is appropriate to consider the current status of transgenic animal technology. This article addresses some of the hurdles that continue to impede application of this technology from solving agricultural problems.

### TRANSGENIC ANIMAL MODEL SYSTEM

Most of the goals currently proposed for genetically altering milk composition rely on gain of function strategies (introduction of a new gene) by means of pronuclear microinjection. However, eliminating or reducing the concentration of specific proteins in milk can also be achieved, at least in theory, by adding new genetic information. By introducing ribozyme or anti-sense genes, translation of specific milk protein genes can be blocked or reduced, thus reducing their protein concentration in milk.

A more widely accepted means of achieving loss of function (eliminating or altering gene function) is through use of gene "knock-out" technology that is dependent on embryonic stem cell. Although outside the scope of this review, development of embryonic stem cells of domestic livestock is being pursued by a number of groups, and embryo-derived pluripotent cell lines have been reported for the pig (81) and cow (71, 73); germline transmission from these cells has not been reported. Recently, lambs were produced following nuclear transfer from an established cell line derived from sheep embryos (31). It remains to be demonstrated whether these cells can be transfected with new genes and selected prior to nuclear transfer and whether such cells can be isolated from other livestock species.

Production of transgenic animals by pronuclear microinjection is practiced today essentially as described in the pioneering first report of Gordon et al. (27), as further characterized by Brinster et al. (11), and as adapted to livestock by our laboratory (33, 78). Table 1 lists the milestones toward produc-

tion of transgenic bioreactors. Interestingly, although the technique has changed little, the definition of the word "transgenic" has taken on new meanings. Jon Gordon and Frank Ruddle originally proposed the term to describe animals in which new genetic material had been introduced by pronuclear microinjection (27). The term has been broadened considerably to include genetically engineered plants, animals produced with the aid of stem cells, and animals that have been subjected to *in vivo* somatic cell engineering (injection of DNA directly into living animals).

### **Efficiency of Producing Transgenic Animals**

The efficiency of producing transgenic animals is low. A compilation of data from several laboratories indicated that about 1 transgenic animal was produced per 40 mouse eggs injected, and the efficiency for sheep, goats, and cattle was much lower, requiring approximately 110, 90, and 1600 egg injections per transgenic animal, respectively (77). Furthermore, only about 50% of transgenic offspring express their transgene. To those working with mice, low efficiency is not of particular concern, but low efficiency is a major impediment to those attempting to produce transgenic livestock. Three parameters account for the low efficiency of the process: embryo survival, gene integration rate, and transgene behavior. In both livestock species and laboratory animals, about 15% of microinjected, transferred embryos survive to term (77). However, gene integration frequency, as measured by proportion of animals born that are transgenic, is much lower for livestock species than for laboratory species (8, 33). That difference in integration rate may be indicative of important biological differences between the zygotes of these species.

### **Transgene Integration Frequency**

The genetic diversity of livestock species may affect the low integration frequency observed. Laboratory animals are derived from highly inbred lines, and investigators often chose to use specific strains for which embryos can be cultured easily. Scientists working with livestock embryos do not have the same inbred resources. However, there are obvious examples in the livestock industry of selection of animals based on gamete quality. One example is the AI industry in which selection of bulls is based not only on genetic merit but also on the freezability of the sperm. Very few researchers have the resources to

perform the same kind of selection on embryo donors.

Another possible cause for the low rate of transgene integration may be related to procedural differences between microinjection of zygotes of livestock and laboratory animals. Livestock eggs are more challenging to microinject than are eggs of mouse, rabbit, or rat. Cow and sow eggs must be centrifuged before microinjection. Although little evidence exists that embryo survival is compromised by centrifugation (78), the procedure may somehow influence integration rate. Within livestock species, the lower integration rate that has been observed for cattle embryos may be partially attributable to the increased difficulty of microinjecting cow eggs. However, that difficulty is not likely to account for the reduction in integration efficiency between cattle and other livestock species.

It is also possible that transgenes become integrated at similar rates but that the development of transgenic livestock embryos and fetuses is disproportionately compromised by transgenes. However, the similar and poor survival rate of both laboratory and livestock embryos does not support that hypothesis. With the data available, it is not possible to determine to what extent susceptibility to transgenes or expression of transgenes accounts for poor livestock embryo survival. Those are probably not a major component (36).

A more compelling argument could be made for an association of integration failure with the inappropriate timing of microinjection. It is well known that transfection of cells in culture is most efficient in dividing populations. Inference from that observation is that DNA replication is required for integration of foreign genes into the genome (4), in which case, the timing of pronuclear microinjection should be synchronized with onset of the DNA S-phase (synthesis phase) of the first cell cycle to ensure the maximum likelihood of an integration event. Mouse eggs are microinjected at about 8 h postinsemination, resulting in DNA being introduced into zygotes during the beginning of S-phase (44). Cow eggs are injected toward the end of the S-phase (23, 24, 40), possibly reducing the probability of an integration event. Pronuclei in cattle zygotes form well before the normal timing of microinjection. However, the normal timing of microinjection is restricted to the time that pronuclei can be visualized in a nondestructive manner (differential interference contrast microscopy). At earlier times, pronuclei cannot be found, even though they are present. Therefore, it would seem to be impossible to microinject transgenes before DNA synthesis is nearly completed in cow eggs. Ex-

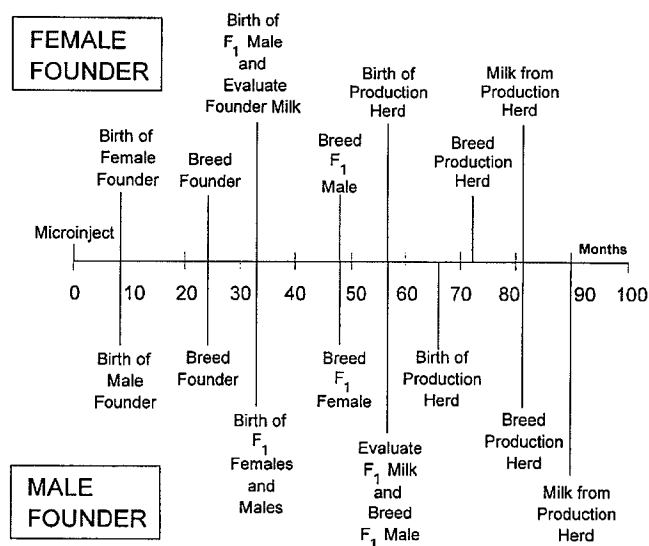


Figure 1. Time line (months) from microinjection to lactation of cows in a transgenic dairy herd arising from a male or female founder. It is assumed that the gestation period is 9 mo, that both males and females reach sexual maturity at 15 mo of age, that the production herd is sired (AI) by a single F<sub>1</sub> male from a line known to express the transgene, and that the first calf of the female founder is male.

perimental evidence is lacking to support the influence of microinjection timing on the frequency of transgene integration, and such evidence may be difficult to achieve. Efforts to inject in vitro fertilized bovine zygotes earlier than 13 h after insemination have failed because of difficulties in visualizing pronuclei and because attempts to manipulate the timing of events during the first cell cycle have been inconclusive.

### Transgene Expression

Characteristics of transgene expression in transgenic goats, mice, pigs, rabbits, rats, and sheep appear to be similar. Although data are insufficient to characterize transgene expression for transgenic cattle, there is no reason to expect that transgenes behave differently in that species. Transgenes appear to integrate randomly in the genome, and approximately half of transgenic animal lines express their transgenes, although some specific transgenes are expressed in a higher proportion of transgenic animals (16, 29). Even in lines that express their transgene, transgene expression is often inappropriate, occurring in unintended tissues (ectopic expression) or at developmentally incorrect times. These aberrant expression patterns or lack of expression have been attributed to the "position effect", which suggests that neighboring genes or heterochromatin regions can

override the control of transgenes. Addition of matrix attachment region sequences or genetic boundary elements to transgene constructs may overcome the position effect in lines that would otherwise carry non-functional transgenes (52).

### Transgene Design and Evaluation

The selection of appropriate regulatory regions, coding sequence type (cDNA versus genomic), introns, and polyadenylation signals for design of mammary specific fusion genes has been severely hindered by the lack of a rapid screening technique. Fusion genes containing promoter regions that are specific to the lactating mammary gland can only be evaluated in lactating mammary epithelial cells. Unfortunately, a transfectable, fully functional system for culturing mammary cells has not been developed. Thus, the labor-intensive and time-consuming transgenic mouse model is viewed as the only reliable technique for evaluation of gene constructs. Even with the relatively short gestation period of the mouse, meaningful evaluation of a lactation-specific construct in F<sub>1</sub> transgenic offspring requires a minimum of 6 to 12 mo. As an initial screening tool, we are developing an in situ transfection technique based on jet injection of naked DNA into lactating mammary glands (25). At 2 d after jet injection of plasmid DNA containing the

human growth hormone (**hGH**) gene, driven by the human cytomegalovirus promoter-enhancer region into mammary glands of lactating sheep, transgene expression was sufficient to be detected by Northern blot analysis (41). We are now using this technique to compare the potencies of various mammary-specific promoter regions in directing expression of the hGH gene (42). Expression is being assessed by both Northern blot analysis and by measurement of hGH protein in tissue extracts. Using the ovine  $\beta$ -LG promoter, up to 40 ng/ml of hGH protein has been detected in the extracts of mammary tissue obtained 48 h postinjection. We are confident that this approach will speed the process of transgene evaluation, serving as a preliminary screening technique prior to the transgenic mouse model, which is much more labor intensive. Finally, because the gene product (protein) can be detected after jet injection of the gene construct, evaluation of posttranslational capabilities of the target tissue in the target species is possible.

#### Time Requirements for Transgenic Animal Projects

Time is by far the greatest resource expended in transgenic livestock projects, as is clearly shown in projects in which the transgene is designed to express only in the lactating mammary gland. In such projects, the minimum time from microinjection to evaluation of milk is equal to twice the gestation length of the target species plus the time from birth to puberty. Once a useful line has been identified, the next step is to collect semen in order to generate a production herd. The time required to achieve these milestones using standard techniques is presented in Figure 1. Clearly, there is a need to develop strategies that shorten the 7 yr to production.

A number of strategies have been demonstrated or

proposed to reduce the time that is required to identify and evaluate transgenic dairy animals as well as the time that is required to expand successful lines (7). For example, expression of a lactation-specific transgene has been detected in milk obtained by artificial induction of lactation in female and male goats (21). More recently, expression of a fusion gene composed of the bovine  $\alpha$ <sub>S1</sub>-CN promoter driving the hGH gene was detected by mammary biopsies obtained 24 h after birth of both male and female rats (37). If such a strategy were proved to reflect transgene expression in adults, then semen from a transgenic bull, collected at approximately 15 mo of age (2 yr following microinjection), could be used to generate a herd producing genetically modified milk in approximately 3 yr—about 5 yr from the time of microinjection.

#### TRANSGENIC ANIMAL BIOREACTORS

A number of companies have been formed within the last decade specifically to exploit transgenic technology. This new industry has been created based on the assumption that production of pharmaceuticals in transgenic animals is more cost effective than production by more conventional means. The US market for bioreactor products thus far identified exceeds \$3 billion/yr (Table 2). All of the products listed in Table 2 are currently derived from human blood, which may account for the fact that the American Red Cross Blood Derivatives Laboratory is one of the leaders in the bioreactor field.

#### Advantages of Transgenic Animal Bioreactors

Almost any living organism, or part thereof, that can be cultivated can serve as a bioreactor. Bacteria, yeast, insect cells, mammalian cells in culture, plants, and chicken eggs are all potential competing produc-

TABLE 2. Estimated annual US requirements and costs of some potential bioreactor products.<sup>1</sup>

| Item                                | Pharmaceutical      |                   |           |                     |            |                       |
|-------------------------------------|---------------------|-------------------|-----------|---------------------|------------|-----------------------|
|                                     | F VIII <sup>2</sup> | F IX <sup>3</sup> | Protein C | AT III <sup>4</sup> | Fibrinogen | Albumin <sup>5</sup>  |
| Estimated quantity needed, kg       | 0.3                 | 4                 | 10        | 21                  | 150        | 315 × 10 <sup>3</sup> |
| Current cost per gram, \$           | 2,900,000           | 40,000            | 10,000    | 7000                | 1000       | 3.56                  |
| Annual market, \$ × 10 <sup>6</sup> | 882                 | 160               | 100       | 150                 | 150        | 1120                  |

<sup>1</sup>Information from William Drohan and Henryk Lubon, American Red Cross (46, 56).

<sup>2</sup>Blood coagulating factor VIII.

<sup>3</sup>Blood coagulating factor IX.

<sup>4</sup>Antithrombin III.

<sup>5</sup>Human serum albumin.

tion systems. Each system has specific advantages and disadvantages. In general, prokaryotic systems and plants can be genetically engineered and propagated rapidly at relatively low cost but lack the mechanisms, or possess the wrong machinery, to perform some of the critical posttranslational modifications (e.g., signal peptide cleavage, glycosylation, amidation, acetylation, carboxylation, and phosphorylation) that are required by complex mammalian proteins (38). Systems for baculovirus-insect cell expression (47) and stable transfected mammalian cells have the capacity to perform authentic posttranslational modifications, but yields in those systems are often an order of magnitude lower than those already achieved in transgenic animal bioreactors.

### **Advantages of the Transgenic Mammary Gland**

The mammary gland is a prodigious production system that is capable of generating between 23 g (dairy cattle) and 205 g (rat) of protein/kg of body weight during peak lactation (55). Milk is clearly the easiest body fluid to collect, especially from ruminants; even pigs can be milked mechanically (30). The ample production capacity of the mammary gland, coupled with the relative ease of harvesting milk in a noninvasive manner, recommends the mammary gland as the organ of choice for producing pharmaceutical products from animals.

Another often cited advantage of producing biologically active products using the mammary gland is the isolation of the mammary gland from the circulatory system. It is argued that bioreactor animals would be protected from the potentially untoward effects of biologically active compounds because those compounds would be sequestered in the mammary gland and therefore would be unavailable to the circulatory system. However, endogenous milk proteins are indeed found in the circulation in cattle, especially during late gestation and at parturition (51), and transgenes and milk protein genes are transiently expressed during estrus, even in virgin mice (64). Therefore, to safeguard bioreactor animals, it may be appropriate to consider designing gene constructs in such a way that their product is converted to an active form after it is isolated from milk.

A potential hurdle to the success of mammary gland bioreactors lies in the ability of the alveolar epithelium to provide appropriate posttranslational modifications such as cleavage of propeptides or signal peptides, N- or O-glycosylation, and  $\gamma$ -carboxylation. Lack of faithful posttranslational modification

of a protein is assumed to affect its biological activity. Furthermore, carbohydrate residues of glycoproteins can serve as antigens, can also influence secretion of a protein, and can affect the half-life of a protein in the circulation (39).

It has recently been shown that, when transgenes for interferon- $\gamma$  were expressed in ovary cells of Chinese hamsters, Sf9 insect cells infected with baculovirus, and mammary glands of transgenic mice, N-glycosylation patterns (sites glycosylated) and composition of the sugar residues differed significantly (39). Glycosylation patterns of human Protein C produced in the mammary glands of transgenic mice (20) or transgenic pigs (54) have been demonstrated to differ from each other and from Protein C isolated from human serum. No additional data are available to assess the significance of these observations on the biological activity of this protein or on the production of other pharmaceuticals in milk. However, at least one prominent laboratory in the bioreactor field is beginning to address the issue by creating transgenic mice that contain two transgenes directed toward the mammary gland, one for the protein of interest and the other to increase the posttranslational modification capabilities of the gland (19).

### **Production Capacity of Transgenic Mammary Glands**

Transgene production capacity is difficult to predict with any certainty. However, from the growing list of studies on transgenic animals in which a milk protein promoter has been used to direct expression of pharmaceutical (Table 3) or milk protein (Table 4) into milk, reasonable production would be at least 1 mg/ml.

The bovine is the target species for transgenic projects aimed at modifying milk for the dairy industry. For bioreactor projects, the target species will be determined on product demand. At an assumed production level of 1 mg/ml, one can calculate the number of animals that would be required to produce some of the proposed pharmaceutical bioreactor products. The results of those calculations are presented in Table 5. On first inspection, it seems unreasonable to think that an organization would consider generating the more than 27,000 rabbits necessary to produce 150 kg of fibrinogen. The labor that is required to maintain and milk those animals would be enormous, especially in light of the fact that 17 cows might be capable of producing all of the fibrinogen required to satisfy current world needs.

TABLE 3. Summary of transgenic animal studies in which a milk protein promoter was used to direct expression of a milk protein into milk.

| Transgenic species | Coding sequence            |            | Promoter region            |            | Protein in milk <sup>1</sup> | Reference |
|--------------------|----------------------------|------------|----------------------------|------------|------------------------------|-----------|
|                    | Gene                       | Source     | Gene                       | Source     |                              |           |
| Murine             | $\alpha$ -LA               | Bovine     | $\alpha$ -LA               | Bovine     | (mg/ml)                      | (5, 70)   |
| Murine             | $\alpha$ -LA               | Caprine    | $\alpha$ -LA               | Caprine    | mg                           | (76)      |
| Murine             | $\alpha$ -LA               | Guinea pig | $\alpha$ -LA               | Guinea pig | ND <sup>2</sup>              | (70)      |
| Murine             | $\beta$ -LG                | Ovine      | $\beta$ -LG                | Ovine      | mg                           | (50)      |
| Murine             | $\alpha$ <sub>S1</sub> -CN | Bovine     | $\alpha$ <sub>S1</sub> -CN | Bovine     | ND                           | (35, 68)  |
| Murine             | $\beta$ -CN                | Bovine     | $\beta$ -CN                | Bovine     | mg                           | (15)      |
| Murine             | $\beta$ -CN                | Bovine     | $\alpha$ -LA               | Bovine     | mg                           | (62)      |
| Murine             | $\alpha$ -CN               | Caprine    | $\beta$ -CN                | Caprine    | mg                           | (6)       |
| Murine             | $\beta$ -CN                | Rat        | $\beta$ -CN                | Rat        | mg                           | (58, 63)  |
| Murine             | $\kappa$ -CN               | Bovine     | $\beta$ -CN                | Caprine    | mg                           | (45)      |
| Murine             | $\kappa$ -CN               | Caprine    | $\beta$ -CN                | Caprine    | mg                           | (31)      |
| Murine             | Lactoferrin                | Human      | $\alpha$ <sub>S1</sub> -CN | Bovine     | mg                           | (57)      |
| Murine             | Lysozyme                   | Human      | $\alpha$ <sub>S1</sub> -CN | Bovine     | $\mu$ g                      | (59)      |
| Murine             | WAP <sup>3</sup>           | Murine     | WAP                        | Murine     | mg                           | (48)      |
| Murine             | WAP                        | Rat        | WAP                        | Rat        | mg                           | (13, 52)  |
| Porcine            | WAP                        | Murine     | WAP                        | Mouse      | mg                           | (2, 16)   |
| Ovine              | WAP                        | Murine     | WAP                        | Mouse      | $\mu$ g                      | (66, 79)  |
| Bovine             | Lactoferrin                | Human      | $\alpha$ <sub>S1</sub> -CN | Bovine     | ND                           | (80)      |
|                    |                            |            |                            |            |                              | (43)      |

<sup>1</sup>Precision of protein determinations varied between publications. Therefore, only the order of magnitude of concentration is given for comparison purposes.

<sup>2</sup>Not determined.

<sup>3</sup>Whey acidic protein.

TABLE 4. Summary of transgenic animal studies in which a milk protein promoter was used to direct expression of a pharmaceutical protein into milk.

| Transgenic species | Coding sequence                    |        | Promoter region            |         | Protein in milk <sup>1</sup> | Reference |
|--------------------|------------------------------------|--------|----------------------------|---------|------------------------------|-----------|
|                    | Gene                               | Source | Gene                       | Source  |                              |           |
| Murine             | $\alpha$ <sub>1</sub> -Antitrypsin | Murine | WAP <sup>2</sup>           | Rabbit  | (mg/ml)                      | (3)       |
| Murine             | $\alpha$ <sub>1</sub> -Antitrypsin | Human  | $\beta$ -LG                | Ovine   | mg                           | (1)       |
| Murine             | $\beta$ -Interferon                | Human  | WAP                        | Murine  | ND <sup>3</sup>              | (65)      |
| Murine             | $\gamma$ -Interferon               | Human  | $\beta$ -LG                | Ovine   | ng                           | (18)      |
| Murine             | CFTR <sup>4</sup>                  | Human  | $\beta$ -CN                | Caprine | $\mu$ g                      | (17)      |
| Murine             | Factor IX                          | Human  | $\beta$ -LG                | Ovine   | $\mu$ g                      | (85)      |
| Murine             | Protein C                          | Human  | WAP                        | Murine  | ng                           | (75)      |
| Murine             | Serum albumin                      | Human  | $\beta$ -LG                | Ovine   | mg                           | (67)      |
| Murine             | Superoxide dismutase               | Human  | $\beta$ -LG                | Ovine   | ng                           | (34)      |
| Murine             | Superoxide dismutase               | Human  | WAP                        | Murine  | mg                           | (34)      |
| Murine             | t-PA <sup>5</sup>                  | Human  | WAP                        | Murine  | ng                           | (28)      |
| Murine             | t-PA                               | Human  | $\alpha$ <sub>S1</sub> -CN | Bovine  | $\mu$ g                      | (61)      |
| Murine             | Trophoblastin                      | Ovine  | $\alpha$ -LA               | Bovine  | $\mu$ g                      | (72)      |
| Murine             | Urokinase                          | Human  | $\alpha$ <sub>S1</sub> -CN | Bovine  | mg                           | (53)      |
| Rabbit             | Interleukin-2                      | Human  | $\beta$ -CN                | Rabbit  | ng                           | (12)      |
| Rabbit             | t-PA                               | Human  | $\alpha$ <sub>S1</sub> -CN | Bovine  | $\mu$ g                      | (61)      |
| Porcine            | Protein C                          | Human  | WAP                        | Murine  | mg                           | (74)      |
| Ovine              | $\alpha$ <sub>1</sub> -Antitrypsin | Human  | $\beta$ -LG                | Ovine   | mg                           | (83)      |
| Ovine              | Factor IX                          | Human  | $\beta$ -LG                | Ovine   | ND                           | (69)      |
| Caprine            | t-PA                               | Human  | WAP                        | Murine  | $\mu$ g                      | (22)      |

<sup>1</sup>Precision of protein determinations varied between publications. Therefore, only the order of magnitude of concentration is given for comparison purposes.

<sup>2</sup>Whey acid protein.

<sup>3</sup>Not determined.

<sup>4</sup>Cystic fibrosis transmembrane receptor.

<sup>5</sup>Tissue plasminogen activator.

However, the required number of rabbits could be produced in 3 to 4 yr by using homozygous males and AI, but 7 to 8 yr would be needed to produce the 17 cows. This comparison is exaggerated but points out the need to consider generation interval as well as production capacity when choosing a species for a bioreactor project.

The data presented in Table 5, admittedly based on preliminary findings, bode well for the practical ability for genetic engineering of mammary glands to produce commercially useful concentrations of foreign protein. Nevertheless, the amount of transgene proteins produced are lower than those of endogenous proteins for all species with the possible exception of sheep. Are the relatively modest concentrations of transgene proteins that have been observed the result of inept transgene design, or are mammary glands already producing close to their maximum capacity? Will milk protein genes have to be knocked out to provide additional capacity for transgene proteins? Will higher transgene product production levels be disruptive to mammary gland function as they were in one study (6)? These questions cannot be answered at this time.

#### MODIFICATION OF MILK TO IMPROVE NUTRITION AND PROCESSING

As mentioned at the outset, a variety of ways have been suggested to modify milk to improve its nutritional quality and to improve the efficiency of manufacturing milk products such as cheese, ice cream, and yogurt (Table 6). Unfortunately, this area of investigation has received less attention in laboratories than it has in review articles.

The limited efforts in this area of research, compared with research in pharmaceutical production,

may reflect the perceived rate of financial return on food products compared with the return on biomedical products. However, a less obvious factor may also have influenced the differences in emphasis of the two fields of genetic engineering. A significant segment of the pharmaceutical industry has embraced the transgenic bioreactor as an approach to making drugs, but little evidence exists that the dairy industry has been as enthusiastic about modifying the type of milk that cows produce. The pharmaceutical industry has a history of obtaining its products from a variety of sources—extracting drugs from plants, synthesizing drugs from chemical constituents, and isolating drugs from animal and human tissues. Isolating these products from the milk of genetically engineered animals does not require a significant change in mind set. However, the prospect of creating cows that produce specialized milk may have momentous consequences for the structure of the dairy industry.

The goal of the dairy industry, at least in the US, has been to create an efficient, healthy cow producing copious amounts of milk that can serve all the needs of the industry. Genetic engineering offers the opportunity for a paradigm shift, a reshaping of the industry from the producers to the processing plants. Dairy producers have the opportunity to choose to produce high protein milk; milk destined for cheese manufacturing that has accelerated curd clotting time; milk containing nutraceuticals, orally administered biologics that provide a health benefit; or a replacement for infant formula. Such a scenario would be a radical change for the dairy industry. But this scenario is by no means without precedent in agricultural production systems. One only has to look to grain, fruit, and vegetable crops to see numerous examples of varieties that have been propagated to fill or create specific niche markets. Whether such a paradigm shift will

TABLE 5. Estimated number of transgenic animals needed to satisfy the annual US market for selected pharmaceuticals.<sup>1</sup>

| Species | Pharmaceutical      |                   |           |                     |                      |                        |
|---------|---------------------|-------------------|-----------|---------------------|----------------------|------------------------|
|         | F VIII <sup>2</sup> | F IX <sup>3</sup> | Protein C | AT III <sup>4</sup> | Fibrinogen           | Albumin <sup>5</sup>   |
| Rabbit  | 54                  | 714               | 1785      | 3750                | 27 × 10 <sup>3</sup> | 56 × 10 <sup>6</sup>   |
| Porcine | 1                   | 10                | 25        | 53                  | 380                  | 800 × 10 <sup>3</sup>  |
| Ovine   | 1                   | 13                | 33        | 70                  | 500                  | 1050 × 10 <sup>3</sup> |
| Caprine | 1                   | 7                 | 17        | 35                  | 250                  | 525 × 10 <sup>3</sup>  |
| Bovine  | 1                   | 1                 | 2         | 3                   | 17                   | 35 × 10 <sup>3</sup>   |

<sup>1</sup>Based on estimated quantity needed (Table 2) and a transgene protein production of 1 g/L.

<sup>2</sup>Blood coagulating factor VIII.

<sup>3</sup>Blood coagulating factor IX.

<sup>4</sup>Antithrombin III.

<sup>5</sup>Human serum albumin.

TABLE 6. Some proposed modifications of milk constituents.<sup>1</sup>

| Change  | Consequence  |
|---|--|
| Increase $\alpha$ - and $\beta$ -CN                       | Enhanced curd firmness for cheese making, improved thermal stability, and increased calcium content.   |
| Increase phosphorylation sites in caseins                 | Increased calcium content and improved emulsification.   |
| Introduce proteolytic sites in caseins                    | Increased rate of textural development (improved cheese ripening).   |
| Increase $\kappa$ -CN concentration                       | Enhanced stability of casein aggregates, decreased micelle size, and decreased gelation and coagulation.   |
| Eliminate $\beta$ -LG                                     | Decreased high temperature gelation, improved digestibility, decreased allergenic response, and decreased primary source of cysteine in milk.      |
| Decrease $\alpha$ -LA                                     | Decreased lactose, increased market potential of fluid milk, decreased ice crystal formation, and compromised osmotic regulation of mammary gland. |
| Add human lactoferrin                                     | Enhanced iron absorption and protected against gut infections.   |
| Add proteolytic sites to $\kappa$ -CN                     | Increased rate of cheese ripening.   |
| Decrease expression of acetyl-CoA carboxylase             | Decreased fat content, improved nutritional quality, and reduced milk production costs.  |
| Express Ig genes  | Protected against pathogens such as salmonella and listeria.   |
| Replace bovine milk proteins genes with human equivalents | Mimicked human breast milk.  |

<sup>1</sup>Adapted (14, 38, 84).

sweep through the entire dairy industry or become a subset of the industry remains to be seen. There is, however, little question that products of genetic engineering will become part of the dairy industry in the next century.

Many of the proposed changes in milk structure are listed in Table 6. It is readily apparent that a number of ways may be used to achieve the same or similar goals. Clearly, altering the characteristics of one component to enhance a particular processing feature may make milk unsuitable for other uses. During the next decade, dairy scientists must determine which approach is most efficacious to achieve the desired goal.

Gibson (26) recently published a thorough analysis of the economic potential of altering milk fat composition and pointed out several potential limitations to that strategy. One consequence of altering milk fat composition would be that increasing the proportion of unsaturated fats (and as a consequence decreasing saturated fats) would increase the melting point of milk, which could impair whipping of cream and increase the hardness of butter. Furthermore, if the alterations resulted in increased free fatty acids, milk rancidity would likely increase. Gibson (26) concluded that the interactive consequences of altering fat content of milk on processing of other milk

products would not be economically advantageous to large segments of the dairy industry, but would only be suitable for niche markets.

However, if the goal is to decrease total fat content rather than to alter the ratios of various fat components, a different economic picture emerges. On average, dairy cows in the US produce milk that contains 3.8% fat. Approximately 50% of that fat is synthesized in the mammary gland. Therefore, if that synthetic machinery in the mammary gland could be disrupted, milk containing 2.0% fat (a 40% reduction) might be achievable (84).

Bremel et al. (9) have suggested that de novo fat synthesis might be reduced by blocking expression of the acetyl-coenzyme A (CoA) carboxylase gene through stem cell ("knockout"), antisense, or ribozyme technology. A recent study (32) confirmed the potential of this concept by transfecting preadipocyte cells with a ribozyme gene directed against acetyl-CoA carboxylase RNA. Acetyl-CoA carboxylase RNA was reduced compared with nonribozyme-expressing cells. The decrease in acetyl-CoA carboxylase mRNA was associated with a significant decrease in activity of acetyl-CoA carboxylase enzyme, and the rate of fatty acid synthesis fell to about 30 to 70% that of controls. This strategy would also have collateral consequences, but might be very cost effective.

Fats are energetically expensive molecules to make, and reducing the fat content of milk to 2.0% could reduce feed energy requirements. Typical dairy rations consist of about 60% forage and 40% concentrate. The cost of forage accounts for about 40% of the cost of feed, and the grain component accounts for about 50% of the feed cost. Engineering of cows to produce 2.0% fat milk would allow the forage component of rations to be increased to 83% (84). The consequence of that change in feed composition would be a reduction of 22% in feed cost per kilogram of milk. Notwithstanding significant savings to the dairy producer, further ramifications could have a positive environmental impact. The amount of forage production would have to be increased by 33%, but the amount of corn produced for dairy rations could be decreased by 50%. Based on corn and forage yields at Beltsville, an acre of land could be taken out of corn production for every 4 cows engineered to produce 2.0% fat milk. Over 2 million acres used for grain production could be reclaimed if all 10 million dairy cows in the US were genetically engineered to produce 2% fat milk. Admittedly, this occurrence is unlikely but demonstrates the potential impact that genetic engineering of dairy cattle could have on other aspects of agriculture.

### CONCLUSIONS

The most easily identified constraint impeding progress in this field is the difficulty in producing useful transgenic animals. As mentioned earlier, low rates of embryo survival and transgene integration have clearly been documented. Because of those two factors, producing a transgenic sheep or goat can easily cost \$60,000, and producing a transgenic cow or bull can exceed \$300,000. The situation is further exacerbated by the unpredictable behavior of fusion gene constructs. Currently, no foolproof method to assess the function of a transgene exists, other than to use it to make transgenic animals of the species of interest. Most organizations test transgene constructs in mice before introducing them into livestock. However, transgenic mice have proved to be poor predictors of transgene function in another species. A way must be found to evaluate transgene constructs accurately in the tissue of the animal of choice without requiring the investment of time and money to produce transgenic livestock. Increased efficiency of producing transgenic animals not only would have the obvious benefit of reducing costs, but also would encourage participation in this field by investigators who cannot currently afford to use this technology.

The participation of other researchers is necessary, especially in the area of modifying milk for postharvesting processing purposes because of the great deal of expertise and research that is required to link the fields of transgenic animal production and dairy food science.

Transgenic animal bioreactor organizations have demonstrated, mostly through empirical studies, that almost any desired protein can be produced in mammary glands. This field of research has now reached the stage of maturity at which the focus of attention will shift to concerns of posttranslational modifications of those proteins, efficacious purification schemes, and product safety. Sixteen years ago, transgenic animal technology was invented, and, since then, an industry has formed to exploit that technology. In the next 16 yr, products created by that technology will likely be in the hands of consumers.

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## Blood Proteins from Transgenic Animal Bioreactors

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**T**HE TRANSMISSION of viruses by blood transfusion and replacement therapy with human plasma proteins has caused serious concern in recent years, eliciting several changes in the preparation of blood products. The selection of donors, rigorous screening of collected blood, and virucidal procedures introduced in the purification of plasma proteins have nearly eliminated the problems of human immunodeficiency virus and hepatitis B virus transmission, and plasma-derived proteins are generally considered to be safe.<sup>1</sup> Past experiences of plasma fractionators with product safety have made them cautious, as shown by the voluntary withdrawal of intravenous immunoglobulin (IGIV) products in 1994 after reports of hepatitis C transmission in Europe, of albumin and factor VIII (FVIII) concentrates containing a unit of blood from a Creutzfeldt-Jakob disease (CJD)-affected donor, and several intramuscular immunoglobulin products in early 1995. The discussion on the contamination of plasma products by infectious agents that could transmit neurodegenerative diseases like CJD or Gerstmann-Sträussler-Scheinker disease, on transfusion-associated bacterial sepsis and parasitic Chagas disease is ongoing.

The production of recombinant human plasma proteins is considered to be an alternative, which will increase the available amount and safety of product. More important, the lower cost of production will permit its use in prophylaxis or for oral administration. As a result, new treatment regimens, such as the topical application of  $\alpha_1$  antitrypsin ( $\alpha_1$ AT) in psoriasis may become feasible. Indeed, the increasing acceptance of recombinant FVIII (rFVIII) by both clinicians and patients has provided added impetus to the search for new sources of plasma proteins. The widespread use of transgenic animals in basic research,<sup>2</sup> development of human disease models,<sup>3</sup> and genetic improvement of livestock<sup>4</sup> have led to considerable interest in employing genetically engineered animals for the production of therapeutic human proteins,<sup>5,6</sup> as an alternative or a complement to eukaryotic cell culture systems. Human plasma proteins being considered for expression are those commonly used in therapy, such as factor IX (FIX),<sup>7,8</sup> FVIII,<sup>9</sup> protein C (PC),<sup>10-11</sup> antithrombin III (ATIII) (H. Meade, personal communication, February 1995), as well

as serum albumin (HSA)<sup>12</sup>,  $\alpha_1$ -AT,<sup>13-14</sup> hemoglobin (Hb),<sup>15</sup> and fibrinogen (Velandar et al: Unpublished observations, February 1994).

In the transgenic approach, synthesis of a recombinant protein is targeted to a selected cell type or organ, enabling the product to be harvested from body fluids like milk, blood, saliva, or urine. Since 1987, numerous proteins with therapeutic potential have been successfully collected by noninvasive methods from transgenic mice or farm animals like rabbits, sheep, pigs, and goats,<sup>16,17</sup> leading to the commercial development of well-established technologies. Questions with respect to transgene inheritance and stability, appropriate posttranslational modifications on heterologous proteins, industrial production procedures, and regulatory affairs have now emerged, as there are limited data published on the long-term effects of foreign protein expression on transgenic animal "bioreactor" (TAB). In this review, the authors discuss the benefits and limitations of transgenic production of human blood proteins, and express their views on its possible impact on transfusion medicine.

### TARGETED EXPRESSION OF TRANSGENES

Recombinant proteins have been targeted to specific tissues of TABs by fusing their DNA coding sequences to the promoters or genomic sequences of homologous or heterologous genes (Fig 1). Since the mid-1980s, expression of blood proteins has been directed to specific organs like the liver, or to cells like erythrocytes and lymphocytes (Table 1). The liver-specific promoters of human  $\alpha_1$ AT,<sup>18-21</sup> apolipoproteins (Apo),<sup>22-24</sup> FIX,<sup>25</sup> and serum amyloid P component genes (SAP)<sup>26</sup> or the erythrocyte-specific globin gene locus,<sup>15,27-30</sup> to name a few, have been used to express the respective genes in transgenic mice. These studies allowed delineation of the regulatory elements of

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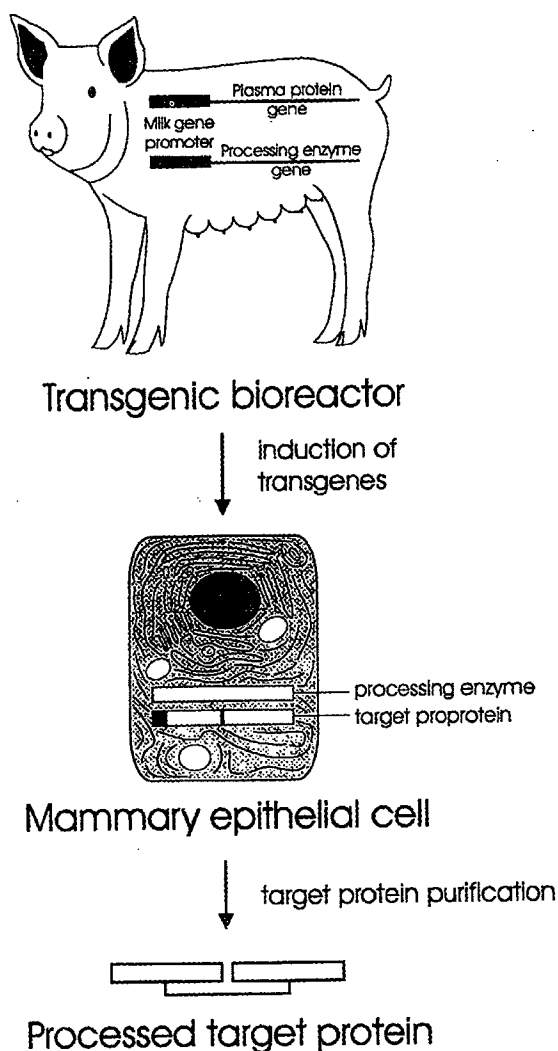


Fig 1. Engineering the mammary gland of a transgenic animal bioreactor. Human plasma proteins are targeted for expression to specific organs like the mammary gland of transgenic animal bioreactors (TABs), using hybrid genes containing the tissue-specific regulatory sequences of milk protein genes and the coding sequences of plasma protein genes. The TAB can be additionally engineered to contain a second transgene encoding the processing enzyme required for efficient posttranslational processing of the target plasma protein. Expression of the transgenes is induced upon lactation and the mature plasma protein is secreted into milk.

plasma protein genes<sup>19</sup> and provided an insight into the molecular basis of disease caused by variant plasma protein genes,<sup>18</sup> as well as models for human disease.<sup>29,30</sup> This also demonstrated the possibility of the production of human plasma proteins in the blood of animals. In practice, however, the expression of active proteins in the bloodstream of animals may not be feasible as high expression levels could be detrimental to health,

and aseptic collection of blood and purification could be difficult. This approach has been followed only for hemoglobin.<sup>15,31</sup>

The regulatory regions of milk protein genes, such as the mouse, rat, and rabbit whey acidic protein (WAP); goat and bovine  $\alpha$ -lactalbumin ( $\alpha$ LAC),<sup>32</sup>; rat, rabbit, and bovine  $\beta$ -casein and sheep  $\beta$ -lactoglobulin ( $\beta$ LG) among others, have been used to direct synthesis of proteins predominantly in the mammary gland. Mice carrying two or more transgenes have also been generated by either mating single transgenics or by coinjection of several transgenes, leading to the assembly of multimeric follicle-stimulating hormone (FSH)<sup>33</sup> and fibrinogen (Velandar et al: Unpublished observations, May 1994). Several human plasma proteins have been secreted into milk by this procedure (Table 2), as have growth factors and hormones (Table 3). Growing evidence of the feasibility of collecting foreign proteins from the milk of TABs has spurred the interest of the American Red Cross and the development of several biotechnology companies, such as Genzyme Transgenics, Framingham, MA; Pharmaceutical Proteins Ltd., Edinburgh, Scotland; and GenePharming Europe, Leiden, The Netherlands.

Today, it is well established that plasma proteins may be expressed in heterologous organs, although it must be noted that some "leakage" or ectopic expression has been observed in other tissues.<sup>12,34-36</sup> New cell or tissue specificities resulting from cooperative effects between transgene regulatory and coding sequences were also observed.<sup>37,38</sup> Some transgenes did not follow the developmental pattern of expression of the endogenous genes. For example, transcripts of recombinant tissue plasminogen activator (rtPA)<sup>35</sup> and protein C (rPC)<sup>36</sup> targeted by the mouse WAP (mWAP) promoter were induced differently from mWAP transcripts during mammary gland development. This altered developmental regulation or ectopic expression<sup>37,38</sup> and the secretion of heterologous plasma proteins into milk did not typically adversely affect the health, nursing ability, or litter size of transgenic animals. Many factors including *cis*-acting elements of gene regulatory regions, intragenic and coding sequences of heterologous genes, and the chromosomal integration site influence the tissue-specific and developmental regulation of transgenes as well as their level of expression. Thus, the pattern of expression of hybrid genes cannot be entirely

Table 1. Blood Proteins Expressed in Body Fluids of Transgenic Mice and Other Animals

| Protein                       | Sequence                               | Regulatory Region                              | Tissue/Fluid                              | Levels (mg/mL)   | Reference No. |
|-------------------------------|--|--|---|------------------|---------------|
| $\alpha_1$ -acid glycoprotein | Gene                                   | Human $\alpha_1$ GP-A, B, C                    | Liver/blood                               | n.a.             | 70            |
| $\alpha_1$ AT                 | Gene                                   | Human $\alpha_1$ AT                            | Liver/kidney/blood                        | n.a.             | 18            |
| $\alpha_1$ AT                 | Gene                                   | Human $\alpha_1$ AT                            | Blood/small intestine                     | 0.1-8.0          | 19            |
| $\alpha_1$ AT                 | Gene                                   | Human $\alpha_1$ AT                            | Liver/kidney/macrophages/<br>blood        | 2.0              | 20            |
| $\alpha_1$ AT                 | Gene                                   | Human $\alpha_1$ AT                            | Blood (rabbit)                            | 1.0              | 21            |
| $\alpha_1$ AT*                | cDNA                                   | mlgk; mlgE <sub>μ</sub>                        | Blood                                     | 0.002-0.01       | 54            |
| Apo A-1                       | Gene                                   | Human Apo A1                                   | Blood                                     | 0.15-2.0         | 22            |
| Apo B100                      | Gene                                   | Human Apo B                                    | Blood                                     | 0.005            | 23            |
| Apo E                         | Gene                                   | Human Apo E                                    | Kidney/liver/blood                        | n.a.             | 24            |
| Apo C-1                       | Gene                                   | Human Apo E                                    | Liver/blood                               | n.a.             | 24            |
| C-reactive protein            | Gene                                   | Human CRP                                      | Liver/blood                               | 0.13-0.35        | 71            |
| FVIII-fragment                | cDNA                                   | m Parotid secretory protein                    | Saliva                                    | 10 units (0.020) | 72            |
| FIX                           | cDNA                                   | mMT1   | Blood                                     | 0.008            | 53            |
| FIX                           | cDNA                                   | mlgk; mlgE <sub>μ</sub>                        | Lymphoid cells                            | 0.001            | 54            |
| FIX                           | cDNA                                   | Human FIX                                      | Hepatocytes/blood                         | 0.0002           | 25            |
|                               | Minigene                               |  |   | 0.006-.04        |               |
|                               | Gene                                   |  |   | 0.007-0.04       |               |
|                               | Gene                                   | Human $\alpha_1$ AT                            |   | .002-.004        |               |
| Hemoglobin                    | $\alpha_1 + \beta$<br>Genes            | h $\beta$ globin LCR                           | Spleen/erythrocytes                       | n.a.             | 27            |
| Hb                            | $\alpha$ Gene<br>$\alpha + \beta$      | h $\beta$ globin LCR                           | Liver/erythrocytes                        | n.a.             | 28            |
| Hb $\beta^s$ , Hb $\alpha_2$  | Genes                                  | h $\beta$ globin LCR                           | Erythrocytes                              | n.a.             | 29            |
| Hb A                          | $\alpha_1, \beta^A$<br>Genes           | h $\beta$ globin LCR                           | Erythrocytes (pig)                        | 1-11 (1%-9%)     | 15            |
| Hb A                          | $\alpha_1, \beta^A, \epsilon$<br>Genes | pig $\beta$ globin + h $\beta$ globin<br>LCR   | Erythrocytes (pig)                        | 32 (24%)         | 31            |
| Hb <sup>Presbyterian</sup>    | $\alpha_1, \beta^P$ Gene               | h $\alpha_1$ globin                            | Erythrocytes (pig)                        | (11%)            | 30            |
| Hb <sup>Yoshizuka</sup>       | $\alpha_1, \beta^Y$ Gene               |  |   | (6.4%)           |               |
| Mouse IgA                     | Gene                                   | Mouse IgA                                      | Blood (mouse)<br>(pig)                    | 0.8<br>0.39-1.38 | 73            |
| IgG <sub>1</sub> , IgM        | Genes                                  | hlgH + ratlgE <sub>H</sub>                     | Blood/lymphocytes                         | 0.001            | 63            |
| Ig-L <sub>K</sub>             |  | hlgL   |   | 1%-20% B cells   |               |
| IgG, IgM                      | Genes                                  | hlgH <sub>μ</sub> + hlgL <sub>K</sub> miniloci | BM/lymphnode/perito-<br>neum/spleen/blood | n.a.             | 64            |
| Ig-L <sub>K</sub>             |  |  |   |                  |               |
| Protein C                     | cDNA                                   | Mouse WAP                                      | Urine                                     | 0.07 μg/mL       | †             |
|                               | Gene                                   |  | Urine (pig)                               | 0.01             |               |
| Serum amyloid Protein         | Gene                                   | Human SAP gene                                 | Liver/blood                               | 0.0004-0.084     | 26            |
| Transthyretin‡ (prealbumin)   | Gene                                   | Human transthyretin gene                       | Liver/blood/yolk sac                      | 0.05             | 74            |

Abbreviations: n.d., not detected; n.a., not assayed; m, mouse; h, human; BM, bone marrow; CRP, C-reactive protein; GP, glycoprotein; IgG, immunoglobulin gamma; IgK or Ig-L<sub>K</sub>, immunoglobulin kappa light chain; IgE<sub>μ</sub>, immunoglobulin μ-enhancer; IgM, immunoglobulin μ; LCR, locus control region; MT1, metallothionein 1, SAP, serum amyloid protein.

\*The Arg<sup>358</sup> "Pittsburgh" variant of  $\alpha_1$  AT, which inhibits thrombin and kallikrein.

†Lubon et al: Unpublished observations, September 1992 (mouse), February 1993 (pig).

‡A Met<sup>30</sup>Val mutant of the human transthyretin gene.

predicted, but understanding of the transcriptional control elements of milk protein and other genes should progress rapidly and will not, in the authors' opinion, hinder TAB technology.

#### EXPRESSION LEVELS

Two types of hybrid genes have generally been used. Transgenes consisting of complementary DNAs (cDNAs) of heterologous proteins generally

gave low expression levels, whereas transgenes containing the genomic sequences were expressed better. In addition, "minigenes" containing the cDNAs and one or more introns have also been used. Expression of cDNA-based hybrid genes was increased by the cointroduction of a second transgene, like endogenous genes with flanking regulatory sequences, to alter the local chromatin structure in the "rescue" approach.<sup>8</sup> Second, "matrix

Table 2. Plasma Proteins Expressed in Milk of Transgenic Animals

| Protein                    | Coding Sequence | Regulatory Region                | Animal | Conc. (mg/mL) | Reference No. |
|----------------------------|-----------------|----------------------------------|--------|---------------|---------------|
| $\alpha_1$ AT              | cDNA            | Sheep $\beta$ LG                 | Mouse  | 0-0.004       | 34            |
| $\alpha_1$ AT + $\beta$ LG | cDNA            | Sheep $\beta$ LG                 | Mouse  | 0.61          | 8             |
| $\alpha_1$ AT              | Gene            | Sheep $\beta$ LG                 | Mouse  | 7.0-12.5      | 13            |
|                            |                 |                                  | Mouse  | 0.4-7.3       | 34            |
|                            |                 |                                  | Sheep  | 1.0-35        | 14            |
| $\alpha_1$ AT*             | Gene            | Rat WAP                          | Mouse  | 10.5          | 44            |
| $\alpha_1$ AT              | Gene            | Goat $\beta$ Casein              | Mouse  | 35.0          | †             |
|                            |                 |                                  | Rabbit | 4.0           |               |
| Antithrombin III           | cDNA            | Goat $\beta$ Casein              | Mouse  | 1.0           | †             |
|                            | cDNA            |                                  | Goat   | 6.0           |               |
|                            | Gene            |                                  | Mouse  | 10.0          |               |
| FVIII                      | cDNA            | Mouse WAP                        | Mouse  | 0.0001        | ‡             |
|                            |                 |                                  | Pig    | n.a.          |               |
| FVIII                      | cDNA            | mWAP; sheep $\beta$ LG           | Mouse  | n.a.          | 9             |
|                            |                 | mMT1; sheep $\beta$ LG           | Sheep  | n.a.          |               |
| FIX                        | cDNA            | Sheep $\beta$ LG                 | Mouse  | n.d.          | 8             |
| FIX + $\beta$ LG           |                 |                                  | Mouse  | 0.001         | 8             |
| FIX                        |                 |                                  | Sheep  | 0.00025       | 7             |
| FX                         | cDNA            | Goat $\beta$ Casein              | Mouse  | 0.7           | †             |
| Fibrinogen                 | cDNAs           | Mouse WAP                        | Mouse  | 0.05          | §             |
| Fibrinogen                 | Gene            | Sheep $\beta$ LG                 | Mouse  | 1.0           | 75            |
| Igs (monoclonal)           | cDNA            | Goat $\beta$ Casein              | Mouse  | 10.0          | †             |
|                            | cDNA            |                                  | Goat   | 5.0           |               |
|                            | Gene            |                                  | Mouse  | 2.0           |               |
| Protein C                  | cDNA            | Mouse WAP                        | Mouse  | 0.003-0.01    | 10            |
|                            | cDNA            |                                  |        | 0.03-0.3      | 61            |
|                            | Gene            |                                  |        | 0.5-0.7       | 48            |
|                            | cDNA            |                                  | Pig    | 0.1-1.0       | 11            |
|                            | Gene            |                                  |        | 0.3-1.8       | §             |
| HSA                        | cDNA            | Sheep $\beta$ LG                 | Mouse  | n.d.          | 12            |
|                            | Minigene 1      |                                  |        | 0.001-0.04    |               |
|                            | Minigene 2      |                                  |        | 2.5           |               |
| HSA                        | Minigenes 3-7   | Sheep $\beta$ LG + SV40 enhancer | Mouse  | 1.0-10.0      | 76            |
|                            | Gene            |                                  |        | 0.01          |               |
| HSA                        | cDNA            | Goat $\beta$ Casein              | Mouse  | 0.8           | †             |
|                            | Gene            |                                  |        | 5.0-10.0      |               |

Abbreviations: n.d., not detected; n.a., not assayed; HSA, human serum albumin; MT1, metallothionein 1.

\*The Ala-357, Arg-358 variant of  $\alpha_1$ AT.

†H Meade: Personal communication, June 1993, February 1994, February 1995.

‡Lubon et al: Unpublished observations, January 1993.

§Velander et al: Unpublished observations, August 1993, February 1994.

attachment sites”<sup>39</sup> and “dominant/locus control regions”<sup>15</sup> have also been successfully used to overcome insertional “position” effects and to increase expression levels in a tissue-specific and developmentally regulated fashion. Finally, the incorporation of hormone-responsive elements, such as glucocorticoid-response elements into transgenes, has also been suggested to obtain inducibility of transgene expression.<sup>40</sup> Isologous promoter replacement was employed to obtain a better ratio of human  $\alpha$ - and  $\beta$ -globin chains in pigs.<sup>31</sup> Expression in founder mice can usually be increased by breeding mosaic animals, segregation of multiple

transgene loci, and establishment of homozygous lines. Some of these principles apply directly to livestock. The secretion of recombinant  $\alpha_1$ AT (r $\alpha_1$ AT), PC (rPC), and HSA (rHSA) at gram per liter levels in milk are good examples of the amount of plasma proteins that can be obtained from TABs (Table 2). For illustration, rPC was secreted at levels 250-fold higher than found in human plasma, 4  $\mu$ g/mL.<sup>11</sup> The authors do not consider the level of expression to constitute a primary limiting factor with TAB technology.

Other questions remain to be answered. Important considerations in the long-term production of

Table 3. Other Therapeutic Proteins Expressed in Milk

| Protein                   | Sequence | Regulatory Region  | Animal | Conc. (mg/mL)   | Reference No. |
|---------------------------|----------|--|--------|-----------------|---------------|
| Erythropoietin            | Gene     | Bovine $\alpha_{s1}$ Casein                                      | Mouse  | n.a.            | 5             |
| Soluble CD4               | cDNA     | Goat $\beta$ Casein  | Mouse  | 5.0             | †             |
| CFTR                      | cDNA     | Goat $\beta$ Casein  | Mouse  | n.a. (fat)      | 77            |
| Glucocerebrosidase        | Gene     | Goat $\beta$ Casein  | Mouse  | 0.1             | †             |
| Growth hormone            | Gene     | Mouse WAP  | Mouse  | 0.41            | 45            |
| Growth hormone            | Gene     | Rabbit WAP   | Mouse  | 4.0-22.0        | 78            |
| Growth hormone            | Gene     | Mouse WAP  | Rat    | 0.007-0.15      | 79            |
| IGF-1                     | cDNA     | Bovine $\alpha$ LAC  | Rabbit | 0.001-4.36      | 80            |
|                           |          | Bovine $\alpha_{s1}$ Casein                                      |        | 0.087-6.48      |               |
|                           |          | Bovine $\beta$ Casein  |        | 0-10.9          |               |
|                           |          | Bovine KCasein   |        | n.d.            |               |
|                           |          | Bovine $\alpha_{s1}$ Casein                                      |        | 0.1-1.0         |               |
|                           |          |  |        | n.a.            |               |
| [Gln <sup>58</sup> ]IGF-1 |          |  |        |                 |               |
| Interferon $\gamma$       | cDNA     | Sheep $\beta$ LG   | Mouse  | 0.4             | 59            |
| Interleukin-2             | Gene     | Rabbit $\beta$ Casein  | Rabbit | 0.43 $\mu$ g/mL | 81            |
| FSH $\alpha$ , $\beta$    | cDNAs    | Rat $\beta$ Casein   | Mouse  | 0.015           | 33            |
| Lactoferrin               | cDNA     | Bovine $\alpha_{s1}$ Casein                                      | Mouse  | 0.036           | 82            |
| tPA                       | cDNA     | Mouse WAP  | Cattle | 2.0             | 6             |
|                           | Gene     |  |        | 30.0            | 6             |
|                           | cDNA     |  |        | n.a.            | 83            |
|                           | cDNA     |  |        | 0.005           | 35, 50        |
|                           | cDNA     |  |        | 0.05            | 84            |
|                           | cDNA     |  |        | 0.05 $\mu$ g/mL |               |
| LAtpA                     | cDNA     | Bovine $\alpha_{s1}$ Casein + Rabbit $\beta$ Globin + SV40 3'seq | Rabbit | 0.05 $\mu$ g/mL |               |
| Urokinase                 | Gene     | Mouse WAP  | Goat   | 0.003           | 42, 51        |
|                           |          | Goat $\beta$ Casein  |        | 3.0             |               |
|                           |          | Bovine $\alpha_{s1}$ Casein                                      | Mouse  | 1.0-2.0         | 85            |

Abbreviations: n.d., not detected; n.a., not assayed; CFTR, cystic fibrosis transmembrane conductance regulator; FSH, follicle stimulating hormone; IGF-1, insulin-like growth factor-1; [Gln<sup>58</sup>] IGF-1, Gln<sup>58</sup> mutated insulin-like growth factor-1; tPA, tissue plasminogen activator; LAtpA, longer acting tPA, an Asn<sup>17</sup>Gln glycosylation variant.

†Meade H: Personal communication, February 1995.

proteins are the stability of the integrated transgene in the germline of animals as well as stable expression of recombinant protein encoded by the transgene over several generations in a line of animals, and over successive lactations in individual animals. Limited information is available on this subject. Aberrant splicing and internal deletion of recombinant FIX (rFIX) transcripts has been observed in the mammary gland.<sup>8</sup> Monitoring the size and sequence of transgene messenger RNA will be necessary to establish transgene and transcript integrity. In  $\alpha_1$ AT transgenic mice, three of four lines studied transmitted the transgene stably, whereas stable lines could not be established with the fourth.<sup>41</sup> Only one of the four lines showed stable expression levels in successive lactations and generations. Transgene expression was turned off in one line, and a 10-fold variation in expression was seen in another line, depending on maternal or paternal transmission. Thus, the genetic background of an animal can influence the level of expression,<sup>41</sup> which may be more consistent in inbred strains

than in F1 hybrids. Conversely, in sheep the  $\alpha_1$ AT transgenes were stably transmitted in three of four lines and less variability in expression was seen, as four founders and one flock of G1 ewes gave similar amounts of  $\alpha_1$ AT in milk in sequential lactations. The output of longer-acting tPA (LAtpA) was constant during lactation, and expression levels were also consistent between lactations in transgenic goats.<sup>42</sup> The amount of recombinant protein secreted on different days of lactation and in successive lactations may affect heterogeneity in terms of posttranslational modifications and extensive studies of the effects of protein on individual animals and in several generations must be performed before setting up a herd for production purposes.

The amount of recombinant protein that can be safely expressed without hurting the animal will have to be determined for every natural or mutated protein and animal species. Secretion of LAtpA at levels of 3 mg/mL, for instance, lead to premature shutdown of lactation in goats because of the

association of LAtPA with  $\beta$ -caseins resulting in insolubility of proteins and nodularity of the mammary gland.<sup>43</sup> The high level expression of  $\alpha_1$ AT in the milk of mice,<sup>13</sup> sheep<sup>14</sup> and rabbits,<sup>44</sup> and of recombinant hemoglobin (rHb) in pig erythrocytes<sup>31</sup> is encouraging because it demonstrates that foreign proteins can constitute up to 50% of the total protein of a body fluid or cell, without obvious deleterious effects. Limitations will be encountered in the amount of heterologous protein expressible, as tissues synthesizing milk, blood, urine, or saliva need to contain some proportion of endogenous proteins for secretion and function. Information on the effects of proteins in blood, secreted either through the basal membrane of the mammary epithelial cells or because of leakage and ectopic expression in other tissues, is rather limited. Leakage may restrict the expression of certain proteins in the TAB as potential deleterious systemic effects can be envisaged for proteins of potent biological activity, such as erythropoietin, tPA, or human growth hormone.<sup>4,45</sup>  $\alpha_1$ AT was detected in blood of sheep at up to 4 g/L, when secreted into milk at high levels of 36 g/L.<sup>46</sup> The potential effects of similar presence of human coagulation factors like rFVIII and rFIX in the blood of TABs are unknown. This emphasizes the fact that fine-tuning of the optimum expression level of each protein in TABs will be necessary.

#### POSTTRANSLATIONAL MODIFICATION OF PROTEINS IN TRANSGENIC ANIMAL "BIOREACTORS"

The biological activity of many proteins is dependent on appropriate posttranslational processing. Low levels of recombinant protein produced by mice did not permit purification and detailed study of their posttranslational modifications in earlier reports. Characterization of proteins synthesized in larger quantities in mice and livestock indicate differences in protein-processing capabilities of TABs compared with human tissue, as discussed subsequently.

##### *Proteolytic Processing of Proproteins*

Proteolytic processing of protein precursors begins with the removal of a hydrophobic signal sequence at or near the aminoterminal. Signal sequences are typically 13 to 30 residues long and are cleaved by signal peptidase during or after translocation of the nascent chain from the endoplas-

mic reticulum. These and other sequences target proteins into the transport pathways by initially directing them to various intracellular compartments. Most sequences exhibit common features and appear to operate in every cell type. It was expected that precursors of plasma proteins generally processed in the human liver would be efficiently cleaved by animal signal peptidases, and available data confirm the correct removal of the signal peptides of rHb<sup>47</sup> in homologous tissue and of  $\alpha_1$ AT,<sup>21,46</sup> rPC,<sup>48,49</sup> rTPA,<sup>35,50</sup> and LAtPA<sup>51</sup> in heterologous organs.

In the Golgi network, proprotein processing is initiated by endoproteolytic cleavage often at the C-termini of paired basic amino-acid residues.<sup>52</sup> The seven known vitamin K-dependent plasma proteins all contain aminoterminal propeptides that direct  $\gamma$ -carboxylation of N-terminal glutamic acid residues and are removed to form the mature proteins. Two of these proteins, Factor X (FX) and PC undergo further proteolytic processing, resulting in the formation of two-chain molecules held together by a single disulfide bond. When FIX transgenes were expressed in the liver of transgenic mice the propeptide was correctly processed.<sup>25,53</sup> However, rFIX produced in the milk of transgenic sheep did not have an electrophoretic mobility similar to plasma-derived FIX, and data regarding propeptide removal were not presented.<sup>7</sup> Similarly, rFIX secreted by transhybridoma cells derived from transgenic mice showed an additional band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and had partial activity,<sup>54</sup> pointing out that lymphoid cells are unable to carry out efficient posttranslational modifications of rFIX. Interestingly,  $\alpha_1$ AT produced in lymphoid cells was glycosylated, of similar molecular weight as human  $\alpha_1$ AT and possessed full biological activity.<sup>54</sup> Studies from the authors' laboratories have demonstrated that rPC in the milk of mice<sup>48</sup> and pigs was incompletely processed.<sup>11,55</sup> Amino-acid sequencing of pig rPC revealed complete removal of the signal peptide after Thr<sup>-24</sup>; however, 20% to 30% of rPC contained the propeptide still attached and 40% to 60% existed in the single-chain form. These data indicate saturation of the endogenous processing protease machinery in the epithelial cells of the mammary gland. Recently, Denman et al<sup>51</sup> demonstrated correct cleavage of the propeptide of LAtPA, at 3  $\mu$ g/mL expression levels, in the mammary gland of a transgenic goat.

LA<sub>1</sub>PA existed mainly in the two-chain form, presumably because of proteolytic processing between Arg<sup>275</sup>-Ile<sup>276</sup> by endogenous milk plasmin after secretion. A minor form of LA<sub>1</sub>PA cleaved between Arg<sup>27</sup>-Ser<sup>28</sup> was also observed, indicating additional cleavage in the mammary gland. rPA secreted into mouse milk was also in the two-chain form, unlike that from Bowes melanoma cells.<sup>35</sup> Conversely, rHSA had electrophoretic mobility similar to HSA,<sup>12</sup> suggesting removal of the proalbumin propeptide and the absence of nonspecific cleavages in mouse milk. However, sequence data of the purified protein would be required to prove this point.

The authors found that rPC synthesized in the pig mammary gland was cleaved at positions -1, 152, and 157 on fractionation of the purified protein,<sup>55</sup> in addition to the expected N-termini at residues -24, 1, and 158. This indicated additional protease activities cleaving at the aminoterminals of Arg residues at the dibasic sites Lys<sup>-2</sup>-Arg, Lys<sup>151</sup>-Arg and Lys<sup>156</sup>-Arg. The newly discovered enzyme cleaving at the N-terminus of arginine in dibasic sites (N-Arginine dibasic convertase)<sup>56</sup> may be responsible for this cleavage and may reflect the increased accessibility of certain sites in foreign proteins to endogenous mammary enzymes.

### Glycosylation

Most plasma proteins under consideration for transgenic production, such as  $\alpha_1$ AT, ATIII, FVIII, FIX, fibrinogen, PC, and tPA are glycoproteins. These proteins have oligosaccharides covalently attached through asparagine (N-linked) or threonine/serine (O-linked) side chains that are important for function. For example, terminal sialic acids appear to protect erythropoietin, tPA, and interferon (IFN) from protease attack, whereas the lack of glycans causes aggregation of fibrinogen and gamma-globulin (IgG).<sup>57</sup> Glycosylation of human plasma proteins proceeds well in TABs; however, subtle differences in oligosaccharide composition and structure have been observed, which could influence the specific biological activity, solubility, rate of secretion, resistance to inactivation and protease attack, plasma clearance, and immunogenicity<sup>58</sup> of recombinant proteins. There is no simple solution to this problem at this time; it can only be tackled by studying the glycosylation pattern of individual heterologous proteins in various livestock species.

$\alpha_1$ AT produced in mouse and rabbit blood was

of the expected molecular weight,<sup>21,54</sup> suggesting complete glycosylation; however, the carbohydrate composition was not analyzed.  $\alpha_1$ AT from sheep milk had a different glycosylation profile,<sup>14</sup> and isoelectric focusing showed 15 to 20 bands with isoelectric-point values ranging from 4.46 to 4.88, unlike human  $\alpha_1$ AT, which resolves between pH 4.42 and 4.67.<sup>46</sup> This increased basicity of  $\alpha_1$ AT may reflect differences in sialylation as well as heterogeneity from the 10% of protein containing a deletion of five N-terminal amino acids. The lack of protective glycosylation at Asn<sup>184</sup>, which inhibits plasmin cleavage of single-chain tPA<sup>57</sup> could have been responsible for rPA in mouse milk being predominantly in the two-chain form.<sup>35</sup> LA<sub>1</sub>PA from goat milk was of lower molecular weight and existed in two glycosylated forms similar to C127 mouse fibroblast cell-derived and human tPA.<sup>51</sup> LA<sub>1</sub>PA contained a population of small, heterogeneous oligosaccharides; had significantly less sialic acid, N-acetylglucosamine (GLcNAc), and galactose (Gal), but contained N-acetylgalactosamine, which is absent in the C127 cell-derived protein. These differences may affect the clearance of LA<sub>1</sub>PA by the plasma asialoglycoprotein receptor. Likewise, although preliminary data showed that rPC showed from pig milk had a similar total carbohydrate content as plasma-derived PC, it also contained N-acetylgalactosamine (GaLNAc), a sugar not observed in PC (Velandar et al: Unpublished observations, September 1991), although detected in 293 human kidney cell-derived rPC. Glycosylation of rPC resulted in increased mobility on SDS-PAGE analysis<sup>48</sup> and considerable species-specific heterogeneity on two-dimensional electrophoresis. A significant contribution to the understanding of this posttranslational modification in TABs was provided by extensive study of recombinant IFN $\gamma$  (rIFN $\gamma$ ) produced in mouse milk.<sup>59</sup> rIFN $\gamma$  showed considerable site-specific variation in glycosylation, with complex sialylated and biantennary core-fucosylated glycans at one N-linked site and mainly oligomannose at the second, which may increase the susceptibility of the protein to clearance by the mannose receptor. The site-specific addition of oligomannose to specific asparagine residues of recombinant ATIII (rATIII) was also observed in the goat mammary gland.<sup>59</sup> Some hybrid N-glycans were also found at the second site, unlike in Chinese hamster ovary (CHO)-cell derived rIFN $\gamma$ . There was no variation in site occu-

pancy of rIFN $\gamma$ , as both sites were completely occupied, in contrast to rIFN $\gamma$  secreted by transfected CHO or *Spodoptera frugiperda* (SF9) cells. This indicated a lack of steric hindrance because of local protein conformation and folding, leading to increased accessibility of the second site in the mammary gland. N-acetylgalactosamine, N-glycolylneuraminic acid, and Gal- $\alpha$ 1,3Gal- $\beta$ 1, 4GlcNAc residues were not detected in transgenic mouse rIFN $\gamma$ , in contrast to proteins from mouse cell lines. The data presented also suggest that the mouse mammary gland may be deficient in the endoplasmic reticulum  $\alpha$ 1-2 mannosidase I and GlcNAc transferases, and the Golgi  $\alpha$ -mannosidase II enzyme levels may be low.<sup>59</sup>

A Gal- $\alpha$ 1,3Gal- $\beta$ 1,4GlcNAc moiety is found on cell surfaces and in secreted proteins of New World monkeys, rodents, pigs, sheep, and cows,<sup>58</sup> and the authors were concerned about its presence on proteins produced in TABs. Given the lack of this moiety on most human cells and proteins and the presence of about 1% of human serum antibodies directed against this epitope, this may affect the half-life of transgenic proteins in circulation and have other immunologic effects. Detailed oligosaccharide structures have not been presented for several glycoproteins produced in TABs, but data on the lack of this moiety on rIFN $\gamma$  is encouraging.<sup>59</sup> As a result, the ideal livestock species for the synthesis of recombinant therapeutic glycoproteins is not yet known. Host cell type, nutrient or enzyme limitations are known to influence processing and site occupancy,<sup>57,58</sup> but the capabilities of the mammary gland are not yet clearly defined. The authors would like to emphasize the complexity of this issue, as understanding of the oligosaccharide composition of human plasma proteins themselves is still growing, evidenced by the recent description of O-fucosylation of residues in the epidermal growth factor (EGF) domain of FIX.<sup>60</sup>

#### *$\gamma$ -Carboxylation*

Vitamin K-dependent blood clotting and regulatory proteins such as prothrombin, Factor VII, FIX, FX, PC, and Protein S require a unique posttranslational modification involving the conversion of glutamic acid residues to  $\gamma$ -carboxyglutamic acid (Gla). Several cell lines have been employed in attempts to produce these proteins, but to the authors' knowledge none have been capable of

synthesizing the amounts of fully  $\gamma$ -carboxylated protein required for economical production. rFIX expressed in mouse liver had activity similar to plasma-derived FIX.<sup>25,53</sup> Similarity has also been demonstrated in electrophoretic mobility, immunorecognition, and Gla content. The authors have observed that the specific anticoagulant activity of rPC purified from the milk of transgenic mice was generally lower than that of plasma-derived PC, 250 U/mg. For example, rPC from mice expressing 10  $\mu$ g/mL had a specific activity of 185 U/mg, whereas rPC from mice expressing 400 to 700  $\mu$ g/mL had an even lower activity.<sup>61</sup> This may be due to saturation of the cellular  $\gamma$ -carboxylase machinery when rPC is overexpressed in the mouse mammary gland, leading to a reduction in the amount of fully  $\gamma$ -carboxylated, biologically active protein. Interestingly, species-specific differences in the ability of mammary epithelial cells to  $\gamma$ -carboxylate heterologous proteins were observed, as overexpression of rPC in the pig mammary gland did not affect  $\gamma$ -carboxylation to the same extent. At expression levels of 0.5 to 1.0 mg/mL, 30% to 60% of rPC was efficiently  $\gamma$ -carboxylated.<sup>11</sup> This may reflect differences in substrate-specificity or enzyme levels between species. No data have been presented on the Gla content of rFIX from sheep,<sup>7</sup> but biological activity suggested that  $\gamma$ -carboxylation may have occurred at 25 ng/mL expression levels.

#### REMODELLING CELLS AND TISSUES OF TRANSGENIC ANIMALS

As discussed earlier, high levels of heterologous protein expression in the mammary gland limit the extent of their proteolytic processing. This may be partially solved by engineering the cleavage sites of proteins to create better substrates for the endogenous endoprotease(s). The tissue content of these enzymes may still pose a limitation. The authors, therefore, decided to genetically engineer the mammary gland by the introduction of processing enzymes under the control of mammary-specific promoters (Fig 1). The first example of this approach is the coexpression of protein C with the paired dibasic amino acid-cleaving enzyme (PACE) or furin.<sup>62</sup> Furin endoproteolytically processes protein precursors at specific pairs of basic amino acids in mammalian cells.<sup>52</sup> Expression of furin in the mammary gland greatly enhanced conversion

of the rPC precursor to its mature form, with cleavage occurring at the correct sites. Amino-acid sequencing confirmed the absence of propeptide and a high percentage of mature protein, even at expression levels of 1 mg/mL.

It follows that other such alterations in the processing machinery of mammary epithelial cells may be attempted. Genes coding for cellular enzymes like the vitamin K-dependent  $\gamma$ -carboxylase and those involved in the synthesis/modification of oligosaccharides on glycoproteins are potential candidates. In fact, "glycosylation engineering" has been carried out in CHO cells to produce specific glycoforms of proteins.<sup>57</sup> The genes could be introduced sequentially or in concert. Thus, the posttranslational capacity of animal organs like the mammary gland may be enhanced and the "bioreactor" remodeled to achieve the protein modifications necessary for biological activity.

Directing the expression of recombinant proteins to secretory organs of animals and collecting protein from the body fluids is one way to produce proteins. Another conception is to synthesize and sequester proteins in easily accessible cells of animals, for example, human hemoglobin in porcine erythrocytes.<sup>15</sup> rHb had a higher oxygen affinity than pig Hb, but the pigs were not anemic. One line of pigs contained 54% of pig-human hybrid rHb, or 25% human rHb and was physiologically normal.<sup>31</sup> With the development of livestock stem cells and homologous gene replacement techniques, the synthesis of human proteins in cells normally containing the host counterpart will become feasible. The production of human antibodies by TABs is also under way and requires even more complex genetic engineering.<sup>63,64</sup> If "humanized" polyclonal antibodies can be synthesized in TABs, this may have a significant impact on the economics and future of the plasma fractionation industry. Another direction for the development of TAB technology is providing a source of novel cells expressing proteins like rFIX,<sup>25,54</sup>  $\alpha_1$ AT,<sup>54</sup> and immunoglobulins.<sup>63,64</sup>

#### PURIFICATION OF TRANSGENIC PROTEINS

The high concentrations of recombinant protein in the body fluids of TABs provide an obvious advantage in improving purity and yield, even though some percentage of the protein may not be fully posttranslationally processed. The expression

of recombinant plasma proteins in tissues where the host counterpart is not expressed makes purification simpler. The conventional processes of protein precipitation and ion-exchange or hydrophobic chromatography, although of lower yield, seem to be effective in isolating a product of acceptable purity. The authors have established the feasibility of purifying rPC from pig milk using selective precipitation by polyethylene glycol, enrichment by barium/citrate precipitation, viral inactivation by solvent/detergent treatment, and fractionation of the active forms by ion-exchange chromatography.<sup>49,55</sup> The rPC was 95% pure and had enzymatic and anticoagulant activities similar to plasma-derived PC. Comparably, more than 95% pure  $\alpha_1$ AT was purified from sheep milk by a process based on ion-exchange, dye affinity, and hydrophobic interaction chromatography, followed by molecular sieving.<sup>14</sup> Immunoaffinity chromatography was used to purify LAtPA from goat milk because of the low expression levels.<sup>51</sup> The high cost of immunosorbents makes them uneconomical for the isolation of hundreds of kilograms of recombinant proteins, although their use may be justified in the production of rFVIII.

The purification of recombinant plasma proteins from milk, urine, blood, saliva, and other tissues of transgenic animals can be complicated by the presence of the corresponding endogenous protein. This is obvious for human plasma proteins secreted into the blood. Milk also contains plasma proteins like immunoglobulins, albumin, antitrypsin, and transferrin that change in concentration during lactation.<sup>65-67</sup> Thus, rHSA produced in the milk of an animal will have to be separated from animal albumin present in milk. Although information in this area is still emerging, the separation of  $\alpha_1$ AT from sheep  $\alpha_1$ AT naturally present at 1 to 2  $\mu$ g/mL in milk was achieved,<sup>14</sup> and human hemoglobin was readily separated from the pig and pig-human hybrid hemoglobins present in pig red blood cells by conventional chromatography to >99% purity.<sup>15,31</sup> However, purification of human antibodies from the milk or plasma of TABs has still to be accomplished.

As oligosaccharides influence solubility and glycoprotein behavior in ion-exchange and hydrophobic chromatography<sup>58</sup> and posttranslational modifications like  $\gamma$ -carboxylation also affect the charge of proteins, the structural differences in plasma

glycoproteins expressed in heterologous tissues will aid in the development of purification protocols separating the recombinant and endogenous protein counterpart. Fractionation of recombinant proteins to obtain glycoforms with enhanced biological activity should also be possible,<sup>55</sup> for example, the less sialylated forms of rPC may be purified for their higher specific activity and potentially shorter circulatory half-life or vice versa.

### CONCLUSION

The cost of producing transgenic founder livestock is estimated to be \$0.25 million to 1.5 million, unlike fermentor facilities that require a minimum investment of \$25 million to 50 million. The operating costs of transgenic animal farms will also be 50% to 75% less than that of cell culture facilities. These basic economic advantages will be the engine for developing the TAB system. At moderate expression levels, just one pig could produce the annual U.S. demand of FVIII, and two pigs would suffice to produce the FIX required (Table 4).

Regulatory concerns about transgenic proteins are admittedly complicated. The U.S. Food and Drug Administration is currently preparing guidelines for transgenic proteins, and the European Commission for Industrial Affairs has recently issued a "Points to Consider" document. Documentation of animal genetic background and health, transgene stability, and characterization of the protein product will be essential. Specific pathogen-free environments or good agricultural practices will have to be established for the maintenance of the herds, protein collection, and purification. Standards for health care and feeding versus grazing will have to be set. The transmission of viruses and other infectious agents within herds, as well as the inactivation of these infectious agents in the final product will have to be addressed. Despite these concerns, it is encouraging to point out that bovine and porcine insulin as well as porcine FVIII have been used in humans for years without evidence of cross-species viral transmission. The authors expect that every therapeutic will have to be evaluated on a case-by-case basis, as the immunogenicity of recombinant protein and possible animal protein contaminants will become especially meaningful with treatment regimens requiring high doses or lifelong administration, depending on the route

**Table 4. Projections of Plasma Protein Production by Transgenic Pigs**

| Plasma Protein | US Requirement (kg/yr) | Expression Level (g/L)* | Transgenic Pigs† |
|----------------|------------------------|-------------------------|------------------|
| Factor VIII    | 0.12                   | 5                       | 1                |
| Factor IX      | 2.0                    | 5                       | 2                |
| Protein C      | 100                    | 5                       | 80               |

\*Estimates assume that the total volume of milk produced by a pig is 500 L/yr.

†Calculations account for a 50% loss during purification from milk.

of administration. Differences in posttranslational modifications of proteins will have to be evaluated with respect to their effects on bioactivity and clinical efficacy. Ethical issues relating to the consequences and metaphysical impact of genetically modified animals,<sup>68</sup> environmental safety issues,<sup>69</sup> and the legalities of patenting transgenic animals are being addressed. Finally, the ultimate benefit to patients will be the critical factor in the success of TAB products.

The authors believe that these issues will be dealt with successfully, albeit over a long time frame, possibly in the first decade of the next century. When this is achieved, human blood proteins from transgenic animals will have a dramatic impact on blood banking, plasma fractionation, and transfusion medicine. The authors expect the impact to be greater on the treatment of isolated deficiencies, whereas the use of plasma fractions in multifactor deficiencies will continue unchanged. It may no longer be necessary for patients to count on donated blood or for surgeons to prepare fibrin sealant of variable quality in hospitals, and effective long-term prophylaxis of hemophilia patients may not cost a fortune.

In August 1995, the FDA Center for Biologics Evaluation and Research, issued document 95D-0131, "Points to consider in the manufacture and testing of therapeutic products for human use derived from transgenic animals," which discusses transgene structure and expression, fidelity of inheritance, and avoidance of contamination by drugs, chemicals, and adventitious agents.

Progress in the expression of FIX was recently reported by J Clark's group. In elegant experiments, they averted the aberrant splicing and deletion of FIX transcripts in the mammary gland by removing a 3' cryptic splice site. This lead to higher level expression of active protein.<sup>86</sup>

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